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# Studies on Acetylcholine Levels in Mouse Brain

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STUDIES ON ACETYLCHOLINE LEVELS IN MOUSE BRAIN

by

THOMAS J. SOBOTKA

A Dissertation Submitted to the Faculty of the Department  
of Pharmacology, Loyola - Stritch School of Medicine in  
Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy

June, 1969

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## BIOGRAPHY

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Thomas Joseph J. Sobotka was born to Mr. and Mrs. Joseph Sobotka on August 16, 1942 in Baltimore, Maryland.

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On August 22, 1964, he joined the former Sharon C. Chandler in holy matrimony.

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## CHAPTER I

### GENERAL INTRODUCTION

The purpose of this introduction is to preface the body of this thesis with a review of some of the evidence for the existence of central cholinergic transmission. The involvement of this cholinergic system with aspects of behavior is the main objective of this thesis.

#### A. THE EXISTENCE OF CHEMICAL TRANSMISSION IN THE PERIPHERAL AND CENTRAL NERVOUS SYSTEMS

Early definitive proof of chemical transmission at nerve terminals is attributed to the findings of Otto Loewi in 1921 (Loewi, 1921). Stimulating the vagal nerves supplying the frog heart released a perfusate which, when reapplied to the heart, caused a cardiac response identical to that caused by vagal stimulation. The active substance in this perfusate, which Loewi called "Vagusstoff," was subsequently identified as a choline ester, probably acetylcholine (Dale and Feldberg, 1934; and Loewi and Navratil, 1926). Loewi's discovery opened a vast field of research which has included every aspect of neurochemical transmission. Abundant evidence established the presence of chemical transmission throughout the peripheral autonomic and neuromuscular nervous system (Eccles, 1964; Koelle, 1965; McLennan, 1963; and Stone, 1957). This peripheral transmission was shown to be mediated by at least two different substances, namely, acetylcholine and norepinephrine (Brown, et al, 1936; Dale, 1933; Dale, et al, 1936; Feldberg and Gaddum, 1934; Laporte and Lorento de No, 1950; and Volle, 1966). The peripheral nervous system was readily amenable to such direct neurohumoral investigation, but similar research in the central nervous system proved to be a much more formidable task.

Compared to the peripheral nervous system, the central nervous system is restrictive in its relatively complex anatomy. This together with the complex and inaccessible synaptic structures of the central nervous system, as well as, some erroneous ideas of the nature of chemical transmission prevented investigators during the first half of this century from obtaining convincing experimental evidence for the existence of central chemical transmission (Eccles, 1964; Mandell and Spooner, 1968; and McLennan, 1963). With the advent of intracellular, micro-electrode recording techniques in 1951 (Fatt and Katz, 1951), the electron microscope in 1954 (De Robertis and Bennett, 1954 and 1955), ultracentrifugation in 1956 (Hebb and Smallman, 1956) and micro-electrophoretic techniques in 1958 (Curtis and Eccles, 1958), electrophysiological and anatomical proof of central chemical transmission began accumulating to the satisfaction of most of the scientific world (Eccles, 1964). However, the molecular nature of the chemical transmitter (s) in the central nervous system remained speculative.

#### B. CRITERIA NECESSARY TO DEFINE A SUBSTANCE AS A CHEMICAL TRANSMITTER

A number of varied substances are constantly increasing the list of proposed central transmitters; for example, acetylcholine, serotonin, catecholamines, histamine, betaine derivatives, Co-enzyme A esters, gamma-aminobutyric acid, substance P, adenosine triphosphate, the cerebellar factor and a vasodilator substance (Aprison, 1962; Crossland, 1962; Dale, 1934; Eccles, 1964; McLennan, 1963; Page and McIsaac, 1962; Quastel, 1962; Roberts, 1962; and von Euler, 1962). One reason for such an extensive list is that various investigators held different opinions and attitudes as to exactly what constitutes adequate proof of a transmitter function.

In an attempt to clarify this confusion, the fulfillment of several criteria has now been accepted as an essential prerequisite to further consideration of a proposed substance as a chemical synaptic transmitter (Eccles, 1964; Mandell and Spooner, 1968; McLennan, 1963; and Reeves, 1966). Generally, the satisfaction of the following six criteria is considered sufficient proof of a transmitter function:

- (1) the proposed substance must be identified as being present in the appropriate neuronal tissues;
- (2) an enzymatic synthesizing system must be demonstrated to maintain a constantly ready supply of this substance;
- (3) a rapidly acting enzymatic system must also be present to destroy the used transmitter substance thereby reconstituting the receptivity of the synaptic region;
- (4) neuronal stimulation should yield an increased output of transmitter; furthermore, periods of rest or neuronal inactivity should result in a decreased output;
- (5) neurons receptive to the suspected transmitter must be demonstrated; and,
- (6) finally, appropriate pharmacological analysis should demonstrate a similarity between exogenously applied and endogenously released transmitter material.

One of the first materials suggested to play a significant role in the central nervous system was acetylcholine (Dale, 1934). Since then, experimental work has established sufficient evidence to satisfy each of the above mentioned criteria.

C. OCCURRENCE OF THE PROPOSED CHOLINERGIC TRANSMITTER IN THE CENTRAL NERVOUS SYSTEM

a. Identity Of The Suspected Cholinergic Transmitter

In 1933 Chang and Gaddum (1933) demonstrated the lack of specificity of any one method for the bioassay of acetylcholine in tissue extracts. However, little attention was paid to this fact and no one really questioned the identity of the cholinergic transmitter as being acetylcholine (Banister, et al, 1953). However, the identification of different types of natural cholinesterases (Alles and Hawes, 1940; and Mendel and Rudney, 1943) and the fact that brain extracts, which has been shown capable of synthesizing acetylcholine (Quastel, et al, 1936; and Stedman and Stedman, 1937), were also capable of synthesizing other cholinomimetic substances (Nachmansohn, et al, 1949), compelled a more careful analysis of the tissue extracts of "acetylcholine" (Banister, et al, 1953). As a result, the latter authors, using chromatographic separation and parallel bioassay techniques, were the first to demonstrate the natural occurrence of homologues of acetylcholine in a biological tissue, namely, ox spleen. Subsequently, cholinomimetic substances other than acetylcholine were also found to be present in brain tissue (Ambache, 1954; and Stone, 1955-b). A recent tabulation of some of these cholinomimetic substances include choline esters, such as acetylcholine, propionylcholine, n-butyrylcholine, and acetyl-B-methylcholine; betaine esters, for example gamma-butyrobetaine; and carnitine derivatives, such as acetylcarnitine (Hosein, et al, 1962; Hosein and Ara, 1962; and Ryall, 1964). All of these substances are

categorized as cholinomimetic, since they possess several important characteristics of cholinomimetic substances in terms of their biological activity on isolated tissues. For example, 1) their activity is qualitatively identical to that of acetylcholine on isolated tissue. 2) Their activity is stable when boiled in an acid solution but rapidly destroyed in an alkaline solution. 3) The biological activity of these substances is usually potentiated in the presence of an anticholinesterase. And 4) Their activity is selectively inhibited by atropine but not by either an antiserotonin or an antihistaminic drug (Aprison and Nathan, 1957-b; Banister, et al, 1953; Delgado and Rubenstein, 1964; Hosein and Ara, 1962; MacIntosh and Perry, 1950; Mitchell, 1963; Quastel, et al, 1936; and Ryall, et al, 1964).

At present the existence of both acetylcholine and other cholinomimetic substances in brain tissue is undisputed. However, the relative importance of these substances in central cholinergic transmission is a subject of considerable ambiguity (Hosein, et al, 1962; Hosein and Ara, 1962; and Ryal, et al, 1964). Until this problem is solved, the possibility remains that the central cholinergic system utilizes several different cholinomimetic substances.

Since this thesis considers the relationship of the central cholinergic "system" to several animal behaviors and is not specifically concerned with the relationship of different possible cholinergic transmitters with such behaviors, the cholinergic material quantitated in this thesis will consist of all of the cholinomimetic constituents recovered in the brain extract. For the sake of simplicity, these

constituents will be referred to, collectively, as "acetylcholine" throughout the paper (unless specifically stated otherwise), with the understanding that no contention is made or inferred that acetylcholine itself is the sole active substance in the unknown extract. Although the exact identity of the active constituents of the brain extract is not established in this research, several accepted tests have been employed to distinguish the cholinomimetic effects on the guinea pig ileum from the non-cholinomimetic actions of histamine, serotonin, substance-P and non-specific interfering factors present in the extract (See Materials and Methods, Chapter III). Much of the past work on the central cholinergic system, to be reviewed in subsequent sections of this thesis, employed criteria similar to those used in this work for identification of the active cholinergic constituents of the extracts (Aprison and Nathan, 1957-b). It should be remembered that these criteria identify merely acetylcholine-like substances and not necessarily acetylcholine itself (Delgado and Rubenstein, 1964; and Mitchell, 1963).

b. Presence And Distribution Of Acetylcholine In The Brain

Since the early finding of acetylcholine in brain tissue by Dikshit (1933-34), steady confirmation through the years solidly established its existence in brain tissue (Aprison, et al, 1968; Crossland, 1951; Crossland and Merrick, 1954; Delgado and Rubenstein, 1964; De Robertis, et al, 1963; Elliott, et al, 1950; Feldberg, 1944-45; Holmstedt, 1967; Hyde, et al, 1949; Kovach, et al, 1957; Malhotra and Pundlik, 1959; Michaelis, et al, 1952; Naruse, et al, 1960; Stone, 1955-a; Toru and Aprison, 1966; and Welsh and Hyde, 1944).

Its occurrence was found not to be restricted to the brains of just one type of mammal, but to occur in many different animals, such as rats (De Robertis, et al, 1963; Giarman and Pepeu, 1964; and Kovach, et al, 1957), rabbits (Aprison and Nathan, 1957-a; Michaelis, 1952; and Tower and Elliott, 1953), mice (the present thesis, see Results; Hano, et al, 1964; Karczmar, et al, 1968; and Kurokawa, et al, 1963), guinea pigs (Beani, et al, 1964; Feldberg, 1944-45; and Ryall, 1964), cats (Dikshit, 1933-34; Elliott, et al, 1950; Feldberg, 1944-45; and Tower and Elliott, 1953), dogs (Feldberg, 1944-45; Malhotra and Pundlik, 1959; and Stone, 1955-a) and even man (Tower, 1955; and Tower and Elliott, 1953). The levels of acetylcholine were reported to range from a fraction of a microgram per gram of tissue to three and four micrograms per gram (McIlwain, 1955; McLennan, 1963; Stone, 1955-a; Tower and Elliott, 1953; and Votava, 1967), roughly decreasing with ascending complexity of behavior, phylogenetically, from mouse to man (Tower and Elliott, 1953). This decrease was related to an increase in average brain weight which in turn resulted from cortical development of non-cholinergic neurons and glia (Feldberg, 1945). In addition to such generic and species differences, strain differences also exist in acetylcholine concentrations of the brain (Bennett, et al, 1960; and see Results of this thesis, Chapter IV).

It is unwise to draw conclusions or make comparisons between the absolute levels of acetylcholine (or any other substance for that matter) determined in different laboratories. A number of factors unique to each laboratory or experiment affect the determination of absolute acetylcholine levels of the brain. For example, two different laboratories might use

different methods of sacrifice, or possibly the extraction procedure or any number of technical parameters may vary — all of which could affect the quantitated level of acetylcholine (Crossland, et al, 1955; and Toru, et al, 1966). Furthermore, the approximate ages and weights of the animals used must be acknowledged, since acetylcholine levels are known to increase with age and/or body weight (Crossland and Merrick, 1954; Feldberg, 1944-45; and Hano, et al, 1964). Rather than admit to such uncertainty in comparing absolute values from different laboratories, it is much more reliable to designate acetylcholine levels of different treated groups relative to a control value determined within the limits of the same experiment, expressing these levels as "percent change from" or "percent of" control. Also, absolute values of different genera can be validly compared when all of these values have been determined within the conditions of the same experimental setting or laboratory. These conditions are rigidly adhered to throughout this thesis.

In contrast to such substances as glucose or oxaloacetic acid, which partake in general cellular metabolism and which are distributed rather randomly throughout the brain with virtually no drastic changes in concentration in any select region, the unequal but persistent distribution of acetylcholine argues for a more specialized function for this substance, such as synaptic transmission (Aprison, 1962). A specialized transmitter function would necessitate the presence of the transmitter predominantly in synaptic areas or, at least, in relation to neuronal cell bodies. This distribution is somewhat characteristic of acetylcholine (MacIntosh, 1941) which, while occurring in both grey and white matter of the brain, was found to predominate in the grey



matter of all regions. However, acetylcholine did occur in significant amounts in such fibrous areas as the corpus callosum, the internal capsule and in the superficial layers of the pons, all of which contain few neuronal cell bodies. Until ultrastructural details of these areas are specifically related, the presence of synaptic terminals in these fibrous regions must remain ambiguous and the possibility of other than a transmitter function for acetylcholine must be considered.

The general anatomical parts of the brain most widely investigated are the telencephalon (including cerebral cortex, hippocampus and basal ganglia), midbrain-diencephalon (including hypothalamus, thalamus and midbrain), pons-medulla and cerebellum. While the highest acetylcholine concentration of the entire brain is in the basal ganglia, (Fink and Urban, 1966; and MacIntosh, 1941), of the regions mentioned above the concentration of acetylcholine is greatest in the midbrain-diencephalon and least in the cerebellum. The telencephalon and pons-medulla are intermediate in concentration with the pons-medulla usually surpassing the former (Aprison, et al, 1968; Barsoum, 1935; Bowers, et al, 1966; Crossland and Merrick, 1954; Dikshit, 1933-34; MacIntosh, 1941; Toru, et al, 1966; Welsh and Hyde, 1944; and see Results of present thesis, Chapter IV).

While, as stated above, comparative levels of acetylcholine may vary between different species of animals, the relationships between regional brain levels of acetylcholine seem to hold true for most, if not all, of the species of animals studied, including man (Aprison, et al, 1968; Barsoum, 1935; Bowers, et al, 1966; Crossland and Merrick, 1954; Dikshit, 1933-34; MacIntosh, 1941; Toru, et al, 1966; and Welsh and Hyde, 1944).

This relationship will also be shown to apply to mouse brain in this thesis (see Results, Chapter IV).

c. Intracellular Location Of The Cholinergic Transmitter

Inherent in any concept of chemical transmission is the necessity for a strategic transmitter location from where it can best carry out its synaptic function upon neuronal stimulation and where it is protected from premature enzymic destruction. The most logical location for a transmitter is in the synaptic region, specifically in the terminal portion of the afferent synapsing nerve. Hebb and Whittaker (1958) in 1958, Whittaker (1959) in 1959 and, later, Ryall (1964) in 1964, using ultracentrifugation, identified a cholinergic substance in a primary "mitochondrial" fraction of rabbit brain tissue in which one major component was nerve endings. They proposed that the acetylcholine of this fraction was embodied in particles envisaged as vesicles of about 0.05 micron in diameter. A deficit in technical ability prevented the establishment of direct evidence for the presence of acetylcholine in such vesicles until 1962 when the laboratory of De Robertis (De Robertis, et al, 1962) developed and applied the method of subfractionation of primary subcellular fractions of rat brain. Using this technique, they were able to separate and isolate the specific components of Whittaker's primary fraction and found vesicular particles, identified by means of the electronmicroscope as similar to Whittaker's proposed synaptic vesicles. Subsequent extraction and bioassay of each of the separate components localized acetylcholine in the component containing synaptic vesicles. The concentration of acetylcholine in the vesicle component was subsequently established for the guinea pig (Whittaker, et al, 1964).

The presence of acetylcholine in these synaptic vesicles not only positions this transmitter material in the most efficient location for carrying out synaptic transmission but it also affords protection to the acetylcholine from enzymic destruction (Whittaker, 1959). Whittaker found that, in the absence of eserine, if the particle-containing fraction was not subjected to any disrupting conditions, the entire amount of acetylcholine, normally recovered in the presence of eserine, is preserved while exogenously added acetylcholine is almost entirely destroyed. Such evidence together with preliminary work by Hebb and Whittaker (1958) and Stone (1955-b) helped to revise some of the earlier notions of the intracellular states in which acetylcholine is found in vivo. It was generally believed that acetylcholine was present in vivo in two forms, one, an inactive, storage form bound to some protein or lipoprotein (Elliott, et al, 1950; and Wilson and Altamirano, 1956) and, second, an active, unbound, free form which consisted of that amount of acetylcholine extracted in the absence of a protein denaturant (Welsh and Hyde, 1944). While the presence of a bound form of acetylcholine was still basically adhered to, the experiments of Whittaker (1959) qualified the definition of bound acetylcholine to include two forms, a stabile and a labile bound form. Only the labile form of the particle-bound acetylcholine is released by relatively mild disruptive treatments, such as ageing at 0°C, osmotic dilution, freezing and thawing, shaking, or cobra venom. But the stabile form is resistant to such mild treatment and therefore requires stronger measures, such as incubation for one hour at 37° or exposure to organic solvents or to pH values of 4 or less.

In describing the conditions that release labile acetylcholine, Whittaker noted that they are conditions which increase permeability of the vesicles without completely damaging them; only complete disruption of vesicle integrity will release the stabile, bound acetylcholine. Since Whittaker proposed this amendment, there has been general agreement that acetylcholine exists in the neuron in the two bound forms and also that "free" acetylcholine of extracted tissues is an artefact and is really representative of the "labile" fraction as defined above (Kurokawa, et al, 1963). A few researchers still ascribe to the presence of free, unbound acetylcholine in vivo, although admittedly, in very small amounts (Sattin, 1966).

#### D. THE CHOLINERGIC SYNTHESIZING SYSTEM

##### a. Identification And Characterization Of Choline Acetylase (ChAc)

The search for a central cholinergic synthesizing system was well underway by 1936, when Quastel, Tennenbaum and Wheatley (Quastel, et al, 1936) demonstrated the ability of rat and guinea pig brain slices to generate a choline ester when incubated in vitro. This ester was subsequently identified as acetylcholine (Stedman and Stedman, 1937). The enzyme responsible for this enzymic acetylation of choline was extracted from brain tissue (not in pure form) by Nachmansohn and Machado (1943) and was called "choline acetylase" by them. They found that the functioning of this enzyme in a soluble system was dependent at least upon the presence of choline and adenosine triphosphate (ATP). Feldberg (1944-45) further characterized this enzyme by demonstrating the necessity of a water soluble, heat-stable co-enzyme for optimal enzymic function. Such a co-enzyme was found by Lipmann not only to be used in

the acetylation of choline but in acylations in general and was referred to as coenzyme A (CoA) (Lipmann and Kaplan, 1946). In such acetylation reactions Lynen (Lynen, et al, 1951) found that acetate reacts with CoA in the presence of some form of energy, such as ATP to form acetyl-CoA, the essential reactive form of acetate, which can then combine with choline in the presence of choline acetylase to form acetylcholine.

It was subsequently found that the formation of acetyl-CoA does not require any specific acetyl donor system but can occur with a variety of donor systems, such as, acetyl phosphate, pyruvate, citrate (Korkes, et al, 1952) or N-acetyl-L-Aspartic acid (Buniatian, et al, 1965) plus their respective enzymes.

Consequent to the discovery of naturally occurring homologues of acetylcholine in biological tissue (Ambache, 1954; Banister, et al, 1953; and Stone, 1955-b) the specificity of brain choline acetylase came under question. On the one hand, Burgen and his colleagues (Burgen, et al, 1956) demonstrated in a very extensive study of rat brain that choline acetylase is not specific for the choline moiety, but can acetylate many other alkyl moieties, especially choline analogues. They further proposed that such may even occur in vivo. On the other hand, Berry and Whittaker (1959) investigated acetate, or acyl-group, specificity. A similar lack of specificity of choline acetylase for the acyl moiety was demonstrated when these authors found that crude brain preparations were capable of synthesizing all choline esters from acetate to hexanoate and also palmitoylcholine.

Therefore, the possibility exists that the in vivo synthesis of cholinergic substances might utilize different acyl and/or alkyl

groupings in specific parts of the brain but not in others. Measurement of the degree of cholinergic synthesis, however, rests upon the presence of some component specific for and common to the entire central cholinergic system; such a component seems to be the coupling enzyme, choline acetylase. Recently, choline acetylase has been interchangeably referred to as choline acetyltransferase (both terms abbreviated ChAc) (Hemsworth and Morris, 1964). The suggestion has been made that choline acetyltransferase represents a family of enzymes (Berry and Whittaker, 1959; and Burgen, et al, 1956) in much the same fashion as cholinesterase represents a family of enzymes (Augustinsson, 1963). If this is found to be true, then an analysis of the regional distribution of specific types of ChAc in the central nervous system would be of great benefit towards a better understanding of the central function of the cholinergic system. However, up to the present, choline acetylase has been treated more as a single enzyme and used as an indicator of the location and concentration of possible cholinergic neurons. The extent of enzymic activity is usually based on the rate of synthesis of acetylcholine per unit of brain tissue in ideal in vitro conditions (Hebb and Smallman, 1956).

b. Distribution Of The Acetylcholine Synthesizing System Within  
The Brain

In view of the unequal distribution of brain acetylcholine, the distribution of brain ChAc is of paramount importance. If the distribution of ChAc follows that of acetylcholine, the requirement of a synthesizing system capable of maintaining a constant supply of acetylcholine, a necessity for a transmitter system, would be fulfilled.

Extensive surveys of the regional distribution of ChAc in the

central nervous system (Feldberg, et al, 1951; Feldberg and Vogt, 1948; Hebb and Silver, 1956; and McCaman and Hunt, 1965) have led to the conclusion that there does exist a linear relationship between the disposition of the enzyme and acetylcholine (Aprison and Takahashi, 1965; Burgen and Chipman, 1951; Feldberg and Vogt, 1948; and Quastel, 1962). Of the regions containing appreciable grey matter, both acetylcholine and ChAc were found to the greatest extent in the caudate nucleus, followed by the thalamus-hypothalamus, pons-medulla, cerebrum and least in the cerebellum (McCaman and Hunt, 1965). However, the presence of ChAc, although relatively inactive, in central white tracts requires explanation, since a transmitter function cannot be ascribed to it in this location. Feldberg and Vogt (1948) suggested that this enzyme is manufactured in the neuronal cell body and transported to the terminals via axoplasmic flow. In support of this idea Hebb (1963) has tabulated evidence implicating the cell body as the sole or chief site of de novo protein synthesis within the neuron. McCaman and Hunt (1965) subsequently proposed calling such axonal ChAc "transient enzyme" en route to the nerve terminals.

Such a general linear relationship of transmitter and synthesizing enzyme was found to hold true across several species of mammals from the rat to man (Hebb and Silver, 1956; and McCaman and Hunt, 1965); and, like acetylcholine, the brain level of ChAc was found to decrease with the ascending order on the phylogenetic scale. The evolutionary development of the neocortex involving multiplication of non-cholinergic neurons and connective tissue (Feldberg, 1945; and Hebb and Silver, 1956) is responsible for this correlation.

c. Intracellular Location Of The Cholinergic Synthesizing System

Hebb and Smallman (1956) initiated the study of the intracellular distribution of ChAc in rabbit brain tissue. The cellular fraction that accounted for most of the enzymic activity was identified as a crude primary mitochondrial fraction. De Robertis (De Robertis, et al, 1963) using rats, also found the primary mitochondrial fraction to contain the greatest ChAc activity. He expanded this finding by subfractionating this primary fraction into its mitochondrial, vesicular and soluble components. Determining not only ChAc activity, but also acetylcholine content, of these three compartments, De Robertis proved a topographical relationship of the transmitter and its synthesizing enzyme within the synaptic complex. Both were found predominantly in the vesicular fraction (consisting of synaptic vesicles) with comparatively minor amounts in the other two compartments.

Although the subcellular distribution of acetylcholine was found relatively constant from species to species (De Robertis, et al, 1963; McCaman, et al, 1965; and Whittaker, et al, 1964), the compartmentalization of ChAc seems highly subject to species variation (McCaman, et al, 1965). Comparing rat, rabbit, guinea pig and pigeon, McCaman found the main variations to be the relative proportion of cytoplasmic or soluble enzyme to vesicular or bound ChAc. (The rat and rabbit had more vesicular ChAc; the guinea pig was intermediate; and the pigeon had mainly cytoplasmic enzyme). Therefore, depending upon the species of animal, acetylcholine and ChAc can be spatially related within the synaptic complex.



In addition to the primary intracellular sites of ChAc associated with nerve terminals, this enzyme is also found in fractions representative of more distant subcellular sites, such as the nuclear and the microsomal fractions (De Robertis, et al, 1963; and McCaman, et al, 1965). This may describe the "transient enzyme" referred to above.

From the above it can be concluded that a system exists for the synthesis and storage of a cholinergic transmitter. Furthermore, this system is present and functional in a location most suitable for the synaptic transmitter, i.e., the nerve terminal.

#### E. INACTIVATION OF THE CHOLINERGIC TRANSMITTER

The presence of a transmitter and its synthesizing enzyme alone does not constitute a complete transmitter system. Needed yet is a rapidly acting enzymic system to reconstitute the synaptic receptor by destroying the released transmitter and to regulate premature activation of the receptor by removing from the synaptic region any excess transmitter.

##### a. Identification And Characterization Of Cholinesterase

The suggestion offered by Dale (1914) in 1914, that there was an enzyme that destroyed acetylcholine preceded an abundance of proof for the existence of not only a single enzyme, but, a family of enzymes capable of this task. The step-by-step discovery of such a family of esterases is fairly completely cited in the reviews of Augustinsson (1948 and 1963) and Whittaker (1951).

The cholinesterases represent a separate family of enzymes distinguished from a wider group of esterases by their unique ability to hydrolyze choline esters (in addition to other esters) and by their

susceptibility to low, specific doses of physostigmine, both of which properties the wider group of esterases do not possess (Augustinsson, 1963). This family of cholinesterases can be subdivided into types on the basis of substrate specificity, enzyme kinetics and specific inhibitors. For the sake of simplicity two major types of cholinesterase have been widely accepted. The first type, called acetylcholinesterase (AChE) by Augustinsson and Nachmansohn (1949), (also referred to as "true," or "specific" cholinesterase and as cholinesterase I) is found in erythrocytes and brain and is a group of enzymes most effective in hydrolyzing those choline and non-choline esters which, structurally, resemble acetylcholine. AChE, is also characteristically inhibited by high concentrations of substrate but is not much affected by low doses of diisopropylphosphorofluoridate (DFP), an organophosphate anti-cholinesterase (Augustinsson, 1963; and Whittaker, 1951).

The second group of cholinesterase, which are found both in blood serum and in brain tissue, have been variably referred to as "pseudo," "non-specific," or "butyro-cholinesterase" and Cholinesterase II or just cholinesterase (pseudo-ChE) (Augustinsson, 1963). These pseudo-ChE's exhibit their maximum hydrolytic activity on those esters resembling butyrylcholine or propionylcholine in structure; their activity is not diminished in the presence of high substrate concentration and they are inhibited, rather selectively, by low doses of DFP (Aldridge, 1953; and Augustinsson, 1963). This group of enzymes represents a large variety of cholinesterases with no clear distinction between them.

Differentiation of these two types of enzymes is hindered by the fact that, while AChE and pseudo-ChE exhibit specificity for their

respective substrates, there is a certain degree of overlapping and each enzyme will hydrolyze the substrate of the other enzyme to a certain degree (Ord and Thompson, 1952). However, the use of selective inhibitors of cholinesterases (Augustinsson, 1948) in conjunction with specific substrates has been the basis of subsequent methods for differentiating between AChE and pseudo-ChE (Ellmann, et al, 1961; Foldes, et al, 1962; and Torack and Barrnett, 1962).

The different types of cholinesterases can be investigated both quantitatively and histochemically. Quantitative determination of the esterases involves incubation of tissue homogenates in the presence of suitable substrates with subsequent measurement of the rate of enzymic hydrolysis (Augustinsson, 1963). The histochemical localization of cholinesterases is based on the technique of incubating tissue sections in the presence of suitable substrate. Treatment of the products of this hydrolysis with suitable reagents renders them visible and fixed at the location of the cholinesterases which can then be inspected microscopically (Koelle and Friedenwald, 1949). Histochemical differentiation of the cholinesterases is possible, using specific substrates and inhibitors (Koelle, 1950) similar to those used for regular quantitative determination.

b. Presence Of The Cholinergic "Degratory" System In The Brain

Quantitative estimates of cholinesterase activity in the brain have shown its distribution is primarily restricted to the grey matter, thereby implying a function related to synaptic-rich or cellular regions (Burgen and Chipman, 1951; Foldes, et al, 1962; McIlwain, 1955; and Pope, et al, 1952). More specifically, cholinesterase is found in

highest amounts in the caudate nucleus; the next highest in the cerebellum, followed by the thalamus, pons-medulla, hypothalamus and cortex all of which contain moderate to low cholinesterase activity (Aprison, et al, 1964; Burgen and Chipman, 1951; Foldes, et al, 1962; and Goldberg and McCaman, 1967).

Of the total brain cholinesterase activity, approximately 8% is attributable to pseudo-ChE (Foldes, et al, 1962; and Koelle, 1954). Only in white matter does pseudo-ChE predominate over AChE (Burgen and Chipman, 1951; Foldes, et al, 1962; and Ord and Thompson, 1952). The relatively high AChE levels of grey matter are subject to some species variation while the low pseudo-ChE levels are more constant (Austin and Phillis, 1965; and Foldes, et al, 1962).

Although it was possible to determine the relative quantities of the different cholinesterases by quantitative, manometric procedures, limitations of these techniques were apparent in that they were incapable of revealing in which cells, or in which parts of cells, the enzymic activities were localized (Holmstedt, 1959). These limitations were lessened by the introduction of the histochemical technique of determining the cellular location of either AChE or pseudo-ChE (Koelle and Friedenwald, 1949). Such histochemical surveys of the cellular distribution of the cholinesterases of the brain (Hebb and Krnjevic, 1962; Holmstedt, 1959; Koelle, 1954 and 1963; and Torack and Barrnett, 1962) identified AChE as the predominant cholinesterase in neuronal cell bodies, axons and terminals of numerous neuronal groups. AChE also was found in some glial cells. In contrast, pseudo-ChE was found principally within neuroglia cells (fibrous astrocytes and perithelial cells), capillaries and other

blood vessels. Except for its rare presence in the nuclear region of some nerve cells, pseudo-ChE is absent from true nervous tissue.

Cholinesterase activity is widespread in the brain. It occurs wherever acetylcholine and choline acetylase are found in the brain in variable amounts. Although the cholinesterase, primarily in the form of AChE, occurs in very large excess (Aprison, 1962; Aprison, et al, 1964; and Russell, 1964) and, therefore, may be found in brain regions which contain no, or rather meager amounts of, acetylcholine or choline acetylase, investigators feel that, in general, there is a fairly good correlation between cholinesterase activity, choline acetylase activity and acetylcholine content in the brain (Eranko, 1967; and Lewis, et al, 1964).

#### c. Intracellular Distribution Of Cholinesterase

Ultracentrifugation studies of brain tissue have revealed that, intraneuronally, cholinesterase is found primarily in those fractions containing membranous material, i.e., crude mitochondrial and microsomal fractions. The nuclear and supernatant fractions contain comparatively little cholinesterase (De Robertis, et al, 1962; and McCaman, et al, 1965). This intraneuronal, cholinesterase consists of AChE, with the exception of the nuclear and supernatant fractions which contain a definite amount of pseudo-ChE (De Robertis, et al, 1962; and Holmstedt and Toschi, 1959).

As previously mentioned, acetylcholine is likewise concentrated in the crude mitochondrial fraction (Whittaker, 1959), but subfractionation of this crude mitochondrial fraction reveals a disparity between the distributions of acetylcholine, ChAc and AChE. While acetylcholine and ChAc predominate in the subfraction containing synaptic vesicles, most

of AChE is associated with membrane-mitochondrial subfraction. Even the small amount of AChE, that is present in the vesicular subfraction, has been shown to be membrane-bound (De Robertis, et al, 1963). This evidence presented by De Robertis favors the view that AChE is in the membranous parts of the synaptic complex in close association with the cholinergic synthesizing unit, the synaptic vesicle.

This completes the identification of the necessary components of the cholinergic transmitter system at the synaptic region: (1) ChAc, present within the synaptic vesicle where it can synthesize and store a ready supply of transmitter; (2) acetylcholine, the cholinergic transmitter; and (3) AChE, associated with synaptic membranes (pre - and/or postsynaptic) to control the amount of active transmitter present in the synaptic region.

#### F. RECOVERY OF NEURONALLY RELEASED TRANSMITTER

One of the previously defined criteria, crucial to the acceptance of acetylcholine as a central transmitter, is the demonstration of a) its release from central structures as a result of nerve stimulation, and b) a lack of appreciable release during neuronal inactivity (Collier and Mitchell, 1966-a and b). As of yet there is no technique available for collecting and identifying chemicals released from single neurons. But with the growing wealth of microtechniques, it might be possible in the near future to analyze single neurochemical events. For example, reverse microiontophoresis might be used to collect material from synaptic regions and the application of gas chromatographic or sensitive bioassay analysis might separate the substances collected. However at present there is no way to obtain direct evidence that acetylcholine (or any other

substance) is released from a nerve terminal as the parikaryon fires (Reeves, 1966).

In reference to acetylcholine release, two different approaches have been used and have been found to compliment each other. The most widely exploited of the two involves comparing brain levels of acetylcholine following varying functional states of activity. This approach is based on the theory that at high levels of neuronal activity the nerves will release a high amount of acetylcholine which after performing its synaptic function is then rapidly destroyed by AChE. Acetylcholine determination at this time should therefore result in lower than normal acetylcholine levels. The opposite would be expected for states of neuronal inactivity, since relatively little transmitter would be released and destroyed (Crossland, 1953; and Richter and Crossland, 1949). Actual experimental data are in agreement with this hypothesis. A measurable decrease in the amount of extractable acetylcholine from brain tissue occurs in association with increased neuronal activation, while an increase in brain acetylcholine occurs during sedation or anesthesia (Crossland, 1953; Crossland and Merrick, 1954; Elliott, et al, 1950; Giarman and Pepeu, 1962; Pepeu and Mantegazzini, 1964; and Richter and Crossland, 1953). The above series of experimental data are in agreement with the findings from the second approach which consists of collecting material directly from the brain surface of the intact animal. The method used was based on the collecting cup technique introduced by MacIntosh and Oborin (1953), and described in detail by Mitchell (1963). The principal of this technique is to maintain a small amount of Ringer

solution, containing eserine, in a small container directly on the surface of a select, small region of the brain. Periodic samples of this solution are taken and assayed for acetylcholine content, thereby yielding a measure of the amount of acetylcholine released by the underlying neural tissue. In the intact brain a spontaneous release of acetylcholine was found to occur from the cortex. Lesions that disrupted the nervous supply to the cortex were found to decrease this spontaneous output (Collier and Mitchell, 1967; and Delgado and Rubenstein, 1964). Excitation of the cortex caused an increase in rate of release of acetylcholine (Collier and Mitchell, 1966-a and b, and 1967; and Mitchell, 1963). Depending upon the extent of neuronal excitation, the rate of release would increase proportionately, from mild sensory or direct electrical stimulation to severe electrically or drug induced seizures (Chakrin, et al, 1968; Collier and Mitchell, 1966-a and b, and 1967; Delgado and Rubenstein, 1964; and Mitchell, 1963). In conscious, free-moving animals it was also shown that the rate of acetylcholine release correlated very well with the state of arousal or behavior activity. An active, aroused animal showed a high rate of release whereas a sleeping, immobile or quiet animal showed a relatively decreased acetylcholine release (Collier and Mitchell, 1966-a and b, and 1967; and Delgado and Rubenstein, 1964). A dramatic demonstration of decreased release was shown upon induction of anesthesia by pentobarbital (Collier and Mitchell, 1966-a and b). The fact that, when the collecting cup was placed over fibrous, white matter, no acetylcholine was recovered, suggests a synaptic origin for the acetylcholine in these experiments (Mitchell, 1963).



Therefore, while specific, direct proof of a neuron to neuron release of acetylcholine is lacking, very convincing indirect evidence is available to substantiate a linear relationship between neuronal activity and rate of transmitter release, in this case acetylcholine.

G. EVIDENCE FOR THE PRESENCE OF CENTRAL CHOLINOCEPTIVE NEURONS

In keeping with the function of a transmitter, the material released from the presynaptic terminal must affect (stimulate or inhibit) the opposing postsynaptic neuron. A cholinergic system must involve cholinceptive neurons in the brain, i.e., neurons receptive to acetylcholine. Cholinoceptivity has been demonstrated by such techniques as injecting compounds into the blood and recording single neurone activity with micro-electrodes (David, et al, 1963; and Marrazzi, 1953) and by implantation or direct injection of cholinergic substances and antagonists into brain regions and observing some physiological parameter, eg. diuresis, eating, sleeping or drinking (Grossman, 1962; Hernandez-Peon, 1962; Miller, 1960; Pickford, 1947; and Pickford and Watt, 1951). The interpretation of the results obtained by these methods have been criticized on the grounds that the observed effects might be due to some indirect action of acetylcholine, eg., an effect on blood supply, glial elements or the blood brain barrier, and thus affect neuronal activity secondarily. Such experiments do not really constitute proof of the existence of central cholinceptive neurons (Curtis and Koizumi, 1961). However, recently, the evidence presented by these methods has been vindicated by studies using micro-iontophoresis (Bradley, et al, 1966; Bradley and Wolstencroft, 1962-a and b; and Curtis and Eccles, 1958). This technique has allowed for the simultaneous application of drugs to

and recording from single central neurons. Acetylcholine has thus been shown to have an excitant action on neurons in the spinal cord (Curtis, 1965; Curtis and Eccles, 1958; and Curtis, et al, 1961), medulla oblongata (Bradley, et al, 1966; and Salmoiraghi and Steiner, 1963), hypothalamus (Bloom, et al, 1963), midbrain (Bradley and Wolstencroft, 1962-a and b), thalamus (Curtis and Andersen, 1962; and Curtis and Davis, 1963), and cerebral cortex (Krnjevic, 1965; Krnjevic and Phillis, 1963; and Spehlman, 1963).

In addition to an excitant action of acetylcholine, inhibitory effects have been found in the midbrain (Bradley and Wolstencroft, 1962-a and b) and in the pons-medulla (Bradley, et al, 1966). Most of these studies uncovered not only acetylcholine sensitive neurons but also a number of neurons insensitive to this substance indicating that while there is a widespread presence of acetylcholine sensitive neurons in the mammalian central nervous system, there also exists other than cholinceptive type neurons.

#### H. PHARMACOLOGICAL ANALYSIS OF CHOLINOCEPTIVE NEURONS

Pharmacological analysis of central cholinceptive neurons has further characterized them. The use of cholinomimetic agents specific for "muscarinic" cholinergic receptors (muscarine, acetyl-B-methylcholine and carbamyl-B-methylcholine) and for "nicotinic" cholinergic receptors (nicotine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and choline phenyl ether) established the presence of both types of receptors on cholinceptive neurons in the medulla and pons (Bradley, et al, 1966) and thalamus (Curtis and Davis, 1963). The nicotinic agents were found to excite the neurons excited by acetylcholine but were without

inhibitory properties. The muscarinic agents mimicked both the excitatory and inhibitory responses to acetylcholine. Confirmation of the presence of these two types of receptors was obtained by using certain cholinolytic agents (Bradley, et al, 1966). Antimuscarinic agents, eg. atropine, blocked some excitatory and all inhibitory effects of acetylcholine, while antinicotinics, eg. dihydro-B-erythroidine, hexamethonium and gallamine, antagonized excitatory responses only.

The effects of pharmacological agents on the central cellular electrophysiological response to both sensory or peripheral electrical stimulation have yielded additional evidence for the presence of an endogenous cholinergic transmitter system. David and his coworkers (David, et al, 1963) found that in the presence of eserine, an anticholinesterase, the cellular response in the lateral geniculate to stimulation of the optic nerve was enhanced. A similar facilitation was noted when a low, subthreshold dose of acetylcholine was given. Atropine, however, was found to depress both the control response and the facilitated response, indicating that a natural cholinergic transmitter was involved. Similarly, Spehlman (Spehlmann, 1963) found that iontophoretically applied acetylcholine increased both spontaneous and light-evoked neuronal firing of visual cortex. (This fact takes on additional meaning when it is remembered that illumination of the eye results in an increased release of acetylcholine from the primary visual cortex (Collier and Mitchell, 1966-a). Spehlman also observed that an anticholinesterase, in the absence of exogenously applied acetylcholine, will activate and facilitate those cells known to be cholinceptive. Marrazzi (1953) made similar observations when he found that an anti-

cholinesterase facilitates transcallosal relay responses between the optic cortices. Such pharmacological analyses have suggested the involvement of endogenous acetylcholine in transmitting nerve impulses across synapses in certain brain areas.

Therefore, all of the essential criteria for a central cholinergic transmitter system have been investigated and satisfactorily fulfilled. However, it is the consensus that other transmitter systems do exist (Aprison, 1962; Aprison and Takahashi, 1965; Carlton, 1963; Crossland, 1962; Eccles, 1964; Feldberg, 1966; Feldberg and Vogt, 1948; Koelle, 1965; Krnjevic, et al, 1966-a, b and c; Reeves, 1966; and Ryall, 1964).

Having thus established the existence of a central cholinergic transmitter system, the main body of this thesis will provide information that could be interpreted to establish relationships between this cholinergic transmitter system and behavior. Three main approaches to this end are presented: 1) The first is a genetic or evolutionary approach which aims at finding a relationship between levels of brain acetylcholine and any of several anatomical, chemical and behavioral parameters measured in several unique genera and strains of mice. 2) In agreement with Russell's (1964) contention that it is of paramount importance to consider the biochemical events associated with behaviors generated from within the animal in response to the environment, the second approach of this paper investigates the changes in acetylcholine levels of specific brain regions as related to environmentally-induced behaviors. 3) The final method of investigation takes the form of the rather classical

consideration of drug-induced changes in acetylcholine levels in discrete brain regions with observations of the behavioral changes that accompany them.

SECTION I

INTERGENERIC WHOLE BRAIN LEVELS OF ACETYLCHOLINE IN  
SEVEN GENERA AND FOURTEEN STRAINS OF MICE

## CHAPTER II

INTRODUCTION TO INTERGENERIC WHOLE BRAIN LEVELS OF ACETYLCHOLINE  
IN SEVEN GENERA AND FOURTEEN STRAINS OF MICE

A. THE PLACE OF GENETIC STUDIES IN BEHAVIORAL RESEARCH

Investigations of the problem of how the central cholinergic system functions to modulate behavior take many forms. All of these approaches correlate the state of functional activity of the cholinergic system with specific behaviors. Little is known of the internal dynamics of the central nervous system and, for the present, at least, it is assumed that a correlation of activity in some subsystem in the brain with an observed behavioral change indicates a causal relationship. On this assumption information can be obtained by inducing changes in the cholinergic system and studying the accompanying behavioral changes, or, by determining alterations in the cholinergic system associated with an experimentally induced behavior (see Section II of this thesis). It is also rational to take advantage of naturally occurring research materials so that, in lieu of introducing non-physiological conditions for the purpose of correlating behavioral and neurochemical changes, one could find among closely related species differences in behavior and chemistry.

Many genetically distinct genera, species and strains of mouse-like rodents have evolved. The genetic makeup of these rodents is related in a unique way with the environment so that each kind of genetically similar animal is defined by a specific, characteristic set of behaviors, anatomical features and neurochemical systems. By proper selection of mice types, one could, in theory, demonstrate between different kinds of rodents a quantitative and qualitative spectrum of any detectable behavior or physiological

parameter, such as, learning, emotionality, motor behavior, exploration, aggression, body weight, brain weight, brain levels of catecholamines, serotonin or acetylcholine, as well as degree of response to drugs or stimuli. All of these parameters, and many more, can be found to varying degrees in different types of mice (Karczmar and Scudder, 1967; Richardson, unpublished data; Scudder, et al, 1969; and see Results of Section I in this thesis). As will be described below, several interesting relationships have already been derived between certain behaviors and neurochemical systems by using mice of differing genera of selective inbreeding. The rationale for using such genetic methods is that, if across the span of genera or strains of mice several parameters, eg. a behavioral trait and a neurochemical system, occur consistently correlated with one another, this might imply a basic genetic and causal relationship between them. However, caution must be exercised in interpreting such genetic correlations. In the first place a genetic relationship of this type does not necessarily represent a real, functional connection but may merely indicate a chance physical presence of unrelated genes at neighboring chromosomal loci. In the second place, assuming a relationship was found to exist between some behavior and a neurochemical system, it is impossible to define this as a primary or a secondary relation until all of the neurochemical systems present can be monitored. The tremendous organizational complexity of the brain makes it highly unlikely that any one behavior is controlled by only one neurochemical system. Rather, an interaction between systems is implied with one transmitter system directly effecting the behavior (primary relation) and the other systems acting as modulators (secondary relation). Aprison, et al (1968) considers the cholinergic system to be the primary



effector with the serotonergic and adrenergic systems as modulators, while Carlton (1963) holds the adrenergic system to be the behavioral activator and the cholinergic system to regulate which behaviors occur and which do not. In any case, however, it is rational, from an evolutionary point of view, that any transmitter system basic to a behavior or essential for the expression of that behavior would be the same and consistently related to its appropriate behavior across genetically diverse, but closely related, groups of mice, while the subtle modulatory systems would be more susceptible to change, qualitatively, from genus to genus. The situation is further complexed by the fact that negative results, e.g. the finding of no correlations in genetic studies, also do not negate a possible basic relationship between the parameters involved (Roberts, 1966). The influence of new or novel genetic systems, present in some animals, might mask a basic mechanism. Also, any selected behavior can quantitatively change depending upon the level of activity of its related neurochemical system, that is to say, the expression of a certain behavior could possibly increase in direct proportion to the activity of its related transmitter system, but only up to a point, beyond which, as the transmitter system increases in activity, the expressed behavior may decline proportionately. This effect is the expected result of a similar phenomenon applicable at any level of physiological activity and known as the inverted-U effect or the optimum theory (Bohdanecky and Jarvik, 1967; Irwin, 1968; Malmö, 1966; Reeves, 1966; and Roberts, 1966). The presence of such a systems property may therefore yield an inverted-U correlation between a behavior and a neurochemical(or physiological) system, rather than a strictly direct or inverse relationship.

Therefore, remembering the limitations of genetic studies, and,

remaining within the proper scope of interpretation, it is realized that they do not define established functional relationships but can only reveal possible correlations and suggest further lines of investigation (Fuller, 1964; and Sudak and Maas, 1964).

B. GENETIC CORRELATIONS OF SEROTONIN AND BIOGENIC AMINES WITH ANIMAL BEHAVIOR

Genetic studies have served to reveal some very interesting relationships between behavior and central biochemical systems. Strains of mice have been found which differ in "emotionality", as measured by the open-field defecation test (Lindzey, 1951), and aggressiveness (Fredericson and Birnbaum, 1954). The strain higher in "emotionality" was found to be lower in aggression and to contain a high brain stem concentration of serotonin (Maas, 1962 and 1963). These findings were confirmed and extended in studies using rat strains (Sudak and Maas, 1964). As with mice, the reactive, more "emotional", rats contained the higher serotonin brain stem concentration. More specifically, these authors were able to measure the limbic serotonin in these rats and found it also to be significantly higher in reactive than in non-reactive animals. Sudak and Maas also found a negative correlation in these rat strains between ambulation (exploratory) scores and brain serotonin, as well as "emotionality" scores. This inverse relationship between ambulation and "emotionality" was previously reported by Maas (1963) and, subsequently, by Broadhurst and Watson (1964). These neurochemical correlations received further verification by the comprehensive genetic studies carried out at the Institute for the Study of Mind, Drugs and Behavior. Not only were interstrain comparisons made, but also, uniquely, intergeneric and interspecies comparisons were carried out. The

different types of mice used provided a continuum of behavioral and neurochemical traits readily amenable to such comparative studies. Many behavioral traits were described to characterize the different types of mice (Karczmar and Scudder, 1966, 1967 and 1968; Karczmar, et al, 1968; Scudder, et al, 1965; Scudder and Karczmar, 1966 and 1967; Scudder, et al, 1966; Scudder, et al, 1967; and Scudder, et al, 1969). The traits most characteristic of the mice were: aggressiveness; degree of exploratory behavior (or ambulation); motor activity; stereotypy; periodic motor immobility ("freezing"); and learning capacity. These mice genera and species were further distinguished by their differing latencies to electroshock seizure (Scudder, et al, 1966). Neurochemically, this selection of mice offered a wide spectrum of differing whole brain serotonin and catecholamine concentrations (Richardson, unpublished data; Scudder, et al, 1966) with which to compare the behavioral traits mentioned. In these studies, the degree of "emotionality" is correlated directly with freezing and stereotypy and inversely with tendencies to explore (Bovet, et al, 1969; Karczmar and Scudder, 1967; Scudder, et al, 1967). In concurrence with previous conclusions drawn from interstrain studies (Maas, 1962 and 1963; Sudak and Maas, 1964), intergeneric comparisons of mice reveal that highly "emotional" states are correlated directly with high brain levels of serotonin (Karczmar and Scudder, 1967; Richardson, unpublished data). These studies also show the brain catecholamines to relate directly with serotonin levels. Furthermore, as reported earlier by Sudak and Maas (1964) and Maas (1962 and 1963), mice genera and species demonstrating low "emotionality" were highly exploratory, exhibited a high degree of motor activity and were also found to be highly aggressive (Karczmar and Scudder,

1967; Scudder, et al, 1967 and 1969). Scott and Fredericson (1951) similarly indicated earlier that there may be a genetic correlation between aggressiveness and less timid behavior or exploration. These latter behaviors of exploration, activity and aggression were associated with low brain levels of serotonin and brain amines. The mice genera showing this fearlessness or lack of timidity appeared better able to learn to avoid the shock than the more emotional mice. Therefore, learning ability seemed here to be related also to low serotonin and catecholamines (Scudder and Karczmar, 1967).

A high degree of correlation was found between increasing brain levels of these same neurochemical substances and increasing electroshock seizure latencies, while no relationship was found to seizure threshold (Scudder, et al, 1966). In agreement with this finding, the laboratory of Schlesinger (Schlesinger, et al, 1965), using strains of mice variably susceptible to audiogenic seizures, also found low serotonin and norepinephrine levels to be more indicative of greater susceptibility to seizure than higher levels. Contrariwise, Lindzey (1951) found highly emotional mice, known to contain high serotonin (Maas, 1962 and 1963), to be more susceptible to audiogenic seizure than less emotional mice.

#### C. GENETIC ASSOCIATION OF LEARNING AND THE CHOLINERGIC SYSTEM

An indication of the complexity of the central nervous system is obtained by considering genetic data reported in the literature recognizing the additional involvement of a cholinergic component in behaviors associated with central biogenic amines and serotonin. The implication of a cholinergic system in intelligence or the ability to solve a problem was based on the fact that rats bred selectively for "maze brightness" and "maze dullness"

(Tryon, 1934) could be differentiated by their brain cholinesterase levels (Bennett, et al, 1958). Subsequently, Rosenzweig (Rosenzweig, et al, 1960) found that in addition to a higher cholinesterase level, the superior learners also had a higher brain acetylcholine content than the inferior strain of rats. To test whether acetylcholine levels and cholinesterase activities are genetically related, rats were specifically bred for high and low cholinesterase (Roderick, 1960) and their acetylcholine levels were compared (Bennett, et al, 1960). Upon observing a lack of correlation between these two parameters, the authors concluded that the genetic mechanisms controlling acetylcholine concentration and cholinesterase activities are independent. However, evidence at hand seems to indicate that the state of cholinesterase activity is dependent in some way on the level of acetylcholine (Reeves, 1966), heightened cholinesterase activity representing a compensatory increase in response to greater acetylcholine production (Rosenzweig, et al, 1968; Tower, 1955; Woolley, et al, 1963). The complex involvement of the cholinergic system in learning was indicated by the findings that nicotine (a typical cholinergic agent) facilitated learning in those strains of mice characterized by low performance levels but tended to depress learning ability in initially high performing strains (Bovet, et al, 1966 and 1969). This type of equalizing effect of nicotine implies an optimum level of activity of the cholinergic system associated with maximum learning ability; an increase in cholinergic activity above the optimum level will result in a decrement in learning. Additional support of cholinergic involvement in learning is found in the fact that, of two strains of mice differing greatly in their learning ability (Bovet, et al, 1969), the better performer contained significantly more brain acetyl-

cholinesterase (Pryor, 1968).

D. GENETIC EVIDENCE ASSOCIATING CHOLINERGIC ACTIVITY AND "EMOTIONALITY"

The only genetic data concerning the relationship of the cholinergic system with "emotionality" was reported by Broadhurst and Watson, (1964). Using rat strains selectively bred for "emotionality", they found a negative correlation between brain cholinesterase activity and high "emotionality". However, little additional pertinent genetic information has since been made available concerning the state of the cholinergic system in relation to the "emotionality" of different genera and strains of animals..

E. THE GENETIC IMPLICATIONS OF CHOLINERGIC INVOLVEMENT IN SEIZURE ACTIVITY

On the basis of their proposition that the level of central cholinergic activity is related to efficiency of neural transmission, Rosenzweig, (1960) suggested that the maze-bright strain of rats, having more brain acetylcholine and cholinesterase, would have a lower convulsive threshold than maze-dull rats. Experimental support for this suggestion was presented by Woolley, et al, (1963). While the maze-bright animals were shown to have lower convulsive thresholds and, therefore, to be indeed, more susceptible to convulsive seizures, compared to the maze-dull strains, their less intense seizure pattern suggested to the authors a lesser reverberation of the seizure current possibly due to the presence of more inhibitory circuits, cholinergic in nature. Using a larger selection of rat strains (6) known to differ in brain acetylcholine and cholinesterase, Woolley, et al, (1963) substantiated their previous findings that rat strains reported to have high brain cholinesterase are associated with low convulsive thresholds. However, seizure thresholds were found to vary

independently of reported acetylcholine concentrations. Also in this expanded study the authors could find no correlation between seizure intensity, post-seizure depression and brain acetylcholine and cholinesterase.

Other genetic data is available supporting the cholinergic system as an important factor in convulsive seizures. Mice strains reportedly highly susceptible to audiogenic seizures (Schlesinger, et al, 1965) were found to have significantly greater brain acetylcholinesterase activity than less seizure-prone strains (Pryor et al, 1966; Pryor, 1968). In addition, convulsive strains of mice were also reported to have higher amounts of brain acetylcholine priming the animal for seizure (Naruse, et al, 1960). Associated with these increased amounts of acetylcholine were similarly increased brain levels of gamma-aminobutyric acid (GABA). Roberts (1960) interpreted this increase in GABA (an inhibitory transmitter) to be a genetically evolved compensation for the increased acetylcholine (the excitatory transmitter). Recently, Rick, et al, (1967) similarly proposed that the cholinergic system might function in some way related to the GABA system in the brain. They based their conclusion on the finding of a very significant correlation between cholinesterase and GABA production in the brains of five strains of rats. Possibly the increased acetylcholine induces a compensatory increase in the synthesis of both cholinesterase and GABA.

As is inferred, there is at present a paucity of data concerning brain levels of acetylcholine in different genera and species of animals. Obviously, before significant meaning can be attached to the proposed functions of the cholinergic system in behavior, as much information as possible must be accumulated about this system. With this end in mind,

the present section of this thesis will present whole brain levels of acetylcholine in several genera, species and strains of mice. The acetylcholine levels will then be compared to the behavioral traits, the neurochemical data and other parameters which characterize each of these mice types. The latter distinguishing traits of the mice were previously determined by other investigators at this laboratory (Karczmar and Scudder, 1967; Richardson, unpublished data; Scudder, et al, 1966; Scudder, et al, 1969; and Scudder and Karczmar, 1967).



## CHAPTER III

MATERIALS AND METHODSA. ANIMALS

The genetic study reported here made use of 7 genera, 9 species and 14 strains of adult, male mice all unique in their ecology, behavior, neurochemistry, neurophysiology, development and pharmacology (Karczmar and Scudder, 1967).

a. Phylogenetic Classification

Phylogenetically, all of these mice are classified on the basis of anatomical similarities in the homologous order Rodentia and sub-order Sciuromorpha (Hall and Kelson, 1959; and Walker, 1964). Within the family Heteromyidae and sub-family Perognathinae is Perognathus longimembris Bailli. In the sub-family Dipodomysinae of the same family are the 2 species of the genus Dipodomys, meriami and deserti.

Reithrodontomys raviventris Raviventris (genus, species and strain, respectively) is classified in the sub-family Cricetinae of the Cricetidae family. Within this same sub-family is found the genus Peromyscus and sub-genus Peromyscus of which 2 species were used in this study, maniculatus and polionotus. Three strains of the maniculatus species were studied, Bairdii, "Colorado" and Gracilis. A third genus, used here, that is included in the Cricetidae family and Cricetinae sub-family is Onychomys, species leucogaster. Within the same Cricetidae family is a second sub-family Microtinae in which Microtus ochrogaster is classified.

The five strains of the genus Mus, species musculus, subspecies domesticus are classified as part of the family Muridae and sub-family Murinae. These strains include four commensals, CF-1, SC-1, ICR and C57BL/6J

and one wild type, "Missouri".

b. Ecology and Development

Ecologically, the albino Mus musculus CF1, SC1, ICR and the black C57BL/6J are laboratory-bred and, if freed, will inhabit buildings and surrounding fields. The wild "Missouri" strain is live-trapped in grassland and field regions of Missouri. Specimens of the genus Mus are omnivorous and can readily adapt to varying food conditions. Their period of gestation ranges from 18 to 21 days. Mus subsequently tends to have a comparatively long period of maturation, growth and behavioral development and remains in the nest under maternal care longer than other small rodents, approximately 3 weeks (Scudder, et al, 1967; and Walker, 1964).

Microtus ochrogaster, the meadow vole, prefers prairie or grassland conditions. It is an herbivorous animal of rather stocky build and is described by some as fearless. After a 21 day gestation period, their maturation and growth is very rapid and weaning occurs at 2 weeks of age (op. cit.).

The genus Onychomys leucogaster, the grasshopper mouse, is, if opportunity permits, a carnivour. It lives under arid to semiarid desert conditions. Unlike Mus, it has a prolonged gestation of 33 days. Also contrary to Mus, Onychomys has a rather rapid rate of maturation and development (op. cit.). Closely related to Onychomys are the 2 species of Peromyscus, polionotus and maniculatus, the 3 strains of this latter species being Bairdii, Gracilis and "Colorado". The genus Peromyscus, also known as white-footed mice or deer mice, can be found in almost every possible habitat within their range. They too, like Mus, are omnivorous. The somewhat prolonged gestation period of 21 to 27 days or longer is followed by a

rather rapid growth and maturation in captivity (Scudder, et al, 1967). However, in the wild maturation seems slower, since young do not disperse from the litter until 3 to 6 weeks after birth (Walker, 1964).

The Reithrodontomys genus, or American harvest mouse, may inhabit salt marshes or tropical forests but are ususally associated with grasslands. Their primarily herbivorous diet occasionally includes insects. The comparatively average 21 to 24 day gestation preludes a long period of maturation. They are weaned at about three weeks of age, similar to Mus (Walker, 1964).

As with Onychomys, Dipodimys meriami and deserti, referred to as kangaroo rats, dwell primarily in arid and semiarid, brush and grass country. This primarily herbivorous mouse propels itself in a unique manner manintaining its balance with its tail. It has one of the longest gestation(4 to 5 weeks and maturation and development(6 weeks) periods of all the mice investigated at this laboratory (Walker, 1964).

Perognathus longimembris Bailli, or desert pocket mouse, inhabits low, arid plains and desert country. Its vegetarian diet is occasionally supplemented with insects, when available. Like their relative Dipodomys, Perognathus undergoes a prolonged gestation period of 3 to 4 weeks (Walker, 1964).

#### c. Behavioral, Neurochemical and Neurophysiological Profiles

Marked behavioral patterns, previously determined at this laboratory, also distinguish these mice, one from the other (Karczmar and Scudder, 1966,1967 and1968; Scudder, et al, 1965; Scudder, et al, 1966; Scudder, et al, 1969; and Scudder and Karczmar, 1966 and 1967). The behavioral apparatus used to quantitate these behaviors included circular photoactivity meters. The initial 15 minute readings were interpreted as

exploratory behavior and subsequent scores were used as motor activity indices. Wheel cages were used to reveal degrees of stereotypic motor activity. Exploration (in addition to photoactivity scores), stereotypy (in addition to wheel cage scores), periodic immobility (freezing) and aggression (also evaluated from data based on isolation induced aggression) were determined using a "mouse city" apparatus, designed at this laboratory by Scudder ( Scudder and Karczmar, 1966 ). This apparatus could quantitate animal behaviors in a field condition and in the presence of other mice. Learning abilities were determined by an automated, hexalevel, electrified climbing screen also designed in this laboratory by Scudder (Scudder, et al, 1965).

The total motor activity of Microtus was shown to be the highest of all the genera investigated. The next, highest activity was exhibited by Mus mice, while Peromyscus and Onychomys demonstrated much lower motor performances. The Mus genus was also characterized by a marked exploratory behavior, followed by Onychomys, at a much reduced level of exploration, then Peromyscus and, finally, Microtus.

Within the Mus genus, the CFL strain showed the greatest exploration followed closely by "Missouri" and, finally, C57BL/6J (Karczmar and Scudder, 1967; and Scudder, et al, 1965).

Stereotypic behavior was most significant in the Peromyscus and lowest in the Mus and Microtus genera. The scoring of stereotypy in "city studies" correlated well with that of wheel-cage activity. Freezing behavior, which is associated with stereotypic responses, similarly predominated in Perognathus and Peromyscus genera but occurred rather rarely in Mus and

Microtus (Karczmar and Scudder, 1967; Scudder, et al, 1965; and Scudder, et al, 1966).

Aggressiveness was virtually absent in the Peromyscus and Onychomys genera but intermediate in Microtus mice studied, while the Mus musculus strains exhibited a relatively high degree (Karczmar and Scudder, 1968; and Scudder, et al, 1969).

Of the genera studied, Mus exhibited relatively good avoidance and learning. Microtus, however, did not show any improvement in performance, while Peromyscus Bairdii and Onychomys did show considerable improvement but never learned to avoid. Perognathus resembled Microtus in its marked inability to learn or improve significantly (Karczmar and Scudder, 1967; and Scudder, et al, 1965). Within the Mus genus, the CFl strain was found to be the best performer in the learning situation, followed by "Missouri" and then by C57BL/6J (op. cit.).

Neurophysiological characterizations of these mice have also been made on the basis of their differential maximum electroshock seizure latencies (Scudder, et al, 1966). In this regard Peromyscus mice were found to have the longest latencies followed by Perognathus, Mus musculus CFl, Mus musculus "Missouri" and Microtus.

Finally, these mice genera are distinguishable on the basis of several neurochemical parameters, namely, serotonin and catecholamine brain concentrations (Figures 7 and 8) (Richardson, unpublished data) and whole brain AChE and total-ChE activities (Figure 6) (Karczmar and Scudder, 1968). The brain differences in acetylcholine (Ach) concentration will be presented in the results of this thesis (Results, Section I).

In summary, therefore, whole brain acetylcholine concentrations as

well as whole brain weights and body weights, were determined for the following kinds of mice: (1) Mus musculus C57BL/6J, CFl, SC1 and "Missouri". This genus is highly aggressive, a rather good performer in learning situations, highly exploratory, a highly active genus and, compared to the other mice, not very timid or "emotional" since they do not display "freezing" (periodic motor immobility) or stereotypic behavior to any appreciable extent. The low electroshock latency of this genus indicates a high susceptibility to seizure. Neurochemically, brain serotonin and norepinephrine are relatively low in this genus and cholinesterase activity is moderate to high; (2) Microtus ochrogaster is described as a fearless animal, not exhibiting "freezing" or stereotypic behavior. It is neither an exploring animal nor is it very aggressive, but it does display a very high degree of motor activity. The performance in learning situations is very poor; of the genera investigated the lowest electroshock latency was displayed by Microtus. This mouse also has low brain serotonin and norepinephrine as well as low cholinesterase activity. (3) Onychomys leucogaster, a virtually unaggressive animal, displays a moderate to low degree of exploratory behavior and learning ability. Its very low motor activity is in distinct contrast to Mus or Microtus mice; Onychomys has very high brain levels of serotonin and norepinephrine while cholinesterase activity is moderate. (4) Peromyscus maniculatus Bairdii, "Colorado" and Gracilis and P. polionotus are generically considered very timid and "emotional", exhibiting marked "freezing" and stereotypic behavior. Also, the very low aggression of this genus compares with their low exploratory activity. While total motor activity is moderately low, Peromyscus demonstrates a moderate degree of learning ability. This genus exhibits the longest latency to electroshock seizure. Neurochemically, serotonin and norepinephrine are present in

moderate to high concentrations as is brain cholinesterase activity;

(5) Reithrodontomys raviventris Raviventris shows the lowest total motor activity of the mice studied. Other behavioral data on this mouse is not yet complete. Chemically, Reithrodontomys has high brain levels of serotonin and norepinephrine, and very high cholinesterase activity;

(6) Dipodomys meriami and deserti have not yet been behaviorally investigated. Neurochemically, however, D. meriami was found to have moderate brain concentrations of serotonin and norepinephrine but very low brain cholinesterase activity; and finally, (7) Perognathus longimembris Bailli, exhibiting very low motor activity, was found to have an intermediate electroshock latency, longer than Mus yet shorter than Peromyscus. High serotonin and norepinephrine brain levels were found to accompany only moderate cholinesterase activity.

In addition to whole brain acetylcholine levels, of the mice described above, acetylcholine concentrations, as well as weights, of brain parts were obtained in four strains of Mus musculus, namely, C57BL/6J, CF-1, SC-1 and ICR.

All mice were given food and water ad libitum. They were maintained in grouped conditions, when possible, in constant temperature and humidity chambers.

For the bioassay of acetylcholine, a guinea pig ileum preparation was used. Male, colored guinea pigs, weighing approximately 300 grams, were obtained from Scientific Small Animal Farm.

## B. EXTRACTION OF ACETYLCHOLINE

### a. Method Of Sacrifice And Excision Of Brain Tissue

Before sacrificing, mice were transferred to the operating room and allowed

to stabilize for about 30 minutes. All mice were sacrificed at approximately the same time of day (11:00 a.m.) to minimize any variations due to circadian rhythm. Each mouse was then weighed and rapidly decapitated using a pair of sharp scissors. Its brain was immediately excised, as rapidly as possible, in the following manner.

A midline incision was made through the skin covering the cranium, thereby revealing the entire dorsal surface of the skull. Inserting the tips of a pair of fine scissors into the opening at the caudal end of the cranium left by decapitation, a dorsolateral cut was made proceeding rostrally first through the cartilage bordering the right side of the brain, then through the left side, both cuts meeting directly over the olfactory bulb region. The cranium was then removed thereby exposing the entire dorsal aspect of the brain. The brain, including olfactory bulbs, was gently lifted out of the skull with a spatula. In the process of removing the brain, the spatula was used to sever the brain from the cranial nerves and the pituitary which were the only elements securing the brain in place within the skull. At this point the brain was either immersed directly into a Dewar flask containing liquid  $N_2$  or divided into parts, depending upon whether the whole brain or parts of brain were to be used. When whole brains were used, the entire process of excision and immersion into liquid  $N_2$  took less than one minute (no discernable variation occurred from genus to genus). In those experiments requiring brain parts, upon removal of the whole brain from the skull, the following dissection was quickly performed. The caudal ends of the dorsal cortex were gently lifted up and forward thereby exposing the telencephalic-diencephalic connections, namely, the striae terminalis and the internae capsulae.



Severing the brain at these points on both the right and left sides separated the telencephalon (T) from the rest of the brain; the telencephalon consisting therefore of the entire cortex, the olfactory bulbs, the hippocampus and the basal ganglia. The midbrain-diencephalon (M-D), which includes the thalamus, hypothalamus and the entire midbrain, was obtained by sectioning the brain at a point immediately rostral to the cerebellum and caudad to the inferior colliculi. Thereafter, removing the third brain part, cerebellum (C), yields the final brain segment, the pons-medulla (P-M) which terminates caudally at a point rostral to the first vertebra. These brain regions were identical to those used by other workers (Aprison, et al, 1968; Toru, et al, 1966), except that the telencephalic region here included olfactory bulbs and a fourth cerebellar region was collected and analyzed in this work. As each of these parts were isolated, they were immediately immersed in vials, containing liquid N<sub>2</sub>, appropriately marked to distinguish the 4 brain parts. This entire process of sacrifice, excision of the brain, division into 4 parts and immersion in liquid N<sub>2</sub> took less than two minutes. Due to the small amount of tissue available per brain, pooling of whole brains and of brain parts was usually found necessary. Therefore, as each mouse was sacrificed and the approximate brain tissue became available, this material was kept frozen in liquid N<sub>2</sub>, until all of the necessary tissue was accumulated. In general, whole brain studies necessitated the pooling of 2 to 3 brains; studies employing brain parts required pooling 4 to 5 brain parts.

Several alternative methods of obtaining brain tissue were considered, to reduce the interval between the death of the mouse and the freezing of its brain. Freezing is the best and most rapid way of minimizing metabolic

activity (Takahashi and Aprison, 1964). All of these methods involved the use of liquid  $N_2$  as the means of initially halting metabolism. Freezing of brain tissue was additionally advantageous in that it has been shown to facilitate recovery of acetylcholine (Crossland, 1951; Stone, 1955-a; and Whittaker, 1959).

An ideal method of obtaining brain tissue is to sacrifice the animal by rapidly freezing it in liquid  $N_2$  or  $O_2$  (Crossland, 1953; and Richter and Crossland, 1949). This was one of the alternative methods studied, but several disadvantages were found to be associated with it. In the frozen state it was very difficult to distinguish brain tissue from other tissue in the head. Also, separation of frozen brain tissue into select regions was found to be technically difficult, not only by this author but also by others (Crossland and Merrick, 1954; Takahashi and Aprison, 1964). In addition, once the brain tissue is frozen it must be maintained this way until the tissue is dispersed into the extraction medium, for even momentary thawing is sufficient to cause serious losses of acetylcholine (Crossland, 1951; Stone, 1955). A more refined technique, developed recently (Takahashi and Aprison, 1964) entails sacrificing the animals in liquid  $N_2$ , as before, but not allowing the brain tissue to freeze, just allowing it to cool to  $0^{\circ}C$ . At this point the animal is removed from the liquid  $N_2$  and placed in a low temperature chamber, where excision and dissection of the brain can be carried out, while maintaining the brain near  $0^{\circ}C$ . While this is a very laudible approach, determination of the critical dipping times into liquid  $N_2$  would have necessitated the use of a large number of each of the different mice types used, since this dipping time would have differed for each, due to their variable head and

brain sizes. This made such an approach untenable, since many of these mice were available in limited supply only.

Another method considered involved decapitating the mouse and subsequently freezing the whole head. This was unacceptable for the same reasons mentioned above.

That the selection of the method used in this thesis was satisfactory is confirmed by the fact that several investigators have found that within two minutes of death very little change in brain acetylcholine will have occurred (Kurokawa, et al, 1963; Stone, 1955-a; and Takahashi and Aprison, 1964).

#### b. Eserinized-Acid-Saline Extraction Procedure

##### i. Solutions

Bicarbonate-free Locke saline with a phosphate buffer was prepared in the following manner (Feldberg, 1944-45). 9.0 grams NaCl, 0.42 gram KCl and 0.24 gram  $\text{CaCl}_2$  were dissolved in enough distilled water to make up 1 liter of solution. To this was added 300 ml. of M/15 sodium phosphate mono-basic buffer (pH about 7.3). Smaller volumes of saline could be made using equivalent concentrations.

Using some of the above Locke saline, a refrigerated stock solution of 0.01% eserine-saline was made by adding an appropriate amount of eserine sulfate.

Two other solutions used during the extraction were N/3 HCl and N/3 NaOH.

All of these solutions were made up fresh two to three times a month.

##### ii. Procedure

Throughout this thesis acetylcholine was extracted from brain tissue (whole brains and brain parts) by a modified form of the eserinizd-acid-saline procedure developed by Feldberg (1943 and 1944-45).

In general, this procedure entailed homogenizing the brain tissue in a chilled extraction solution consisting of 4 ml. of eserine-saline and 0.5 ml. of N/3 HCl. Subsequently, the homogenate was heated in a boiling water bath for 10 min., cooled, centrifuged and the supernatant collected. At this point the extract was determined to have a pH of 3-4 (pH was approximated using phydron paper). After neutralizing with N/3 NaOH, the extract was either assayed immediately or frozen and assayed the following day. On several occasions neutral solutions of acetylcholine were prepared and their activity tested at intervals for several days after freezing. No loss in acetylcholine activity was detected, even after freezing for 3 days.

The presence of eserine in the extracting medium was of importance to nullify the effect of the cholinesterase present (Feldberg, 1943; and Stone, 1955-a). However, it seems that eserine might merely serve as a precautionary measure, since several investigators have found that the acid in the extract is capable of inactivating cholinesterase (Elliott, et al, 1950; and MacIntosh and Perry, 1950).

The combination of heating brain tissue in the presence of acid serves several functions most important for an accurate extraction of tissue acetylcholine. As mentioned previously, acetylcholine is found in a labile and stabile form. The labile acetylcholine is readily released by relatively mild treatment such as mechanical disintegration or freezing and thawing (both treatments, as described are

employed here). But the stabile acetylcholine requires stronger measures for its release, e.g. heating or acid treatment (Whittaker, 1959).

Therefore, in accord with Whittaker, as well as many other investigators, (Feldberg, 1945; Kurokawa, et al, 1963; MacIntosh and Perry, 1950; and Ryall, et al, 1964), acid treatment and heat treatment were both used to liberate all of the acetylcholine present, both in the labile and stabile forms, and to obtain maximum yield.

Homogenized brain tissue is well known to retain its capacity to synthesize new acetylcholine (Hemsworth and Morris, 1964). The presence of such a functioning synthesizing system would render any determinations of brain acetylcholine levels of questionable validity. Such an interfering factor is obviated here by the presence of acid in the extraction media. The synthesizing ability of choline acetylase is inhibited in the presence of acid and subsequent heating results in denaturation of this enzyme system (Feldberg, 1943 and 1950; Giarman and Pepeu, 1964; Hemsworth and Morris, 1964; MacIntosh and Perry, 1950; and Toru and Aprison, 1966).

An additional benefit accrued from the presence of HCl in the extracting media is that stability in an heated acid solution is one of the criteria used to identify the presence of a choline ester (Banister, et al, 1953; Elliott, et al, 1950; and Stone, 1955-b). This is one of three criteria used in the present paper.

Other methods of extraction are available, entailing the use of such solvents as trichloroacetic acid (Crossland, 1951), acid-alcohol (Stone, 1955-a) or formic acid-acetone (Toru and Aprison, 1966) and of reineckate precipitation (Bentley and Shaw, 1952). However, in the opinion

of this author these procedures offer little added advantage over the present method of extracting acetylcholine.

More specifically, this eserinizid-acid-saline method of extracting acetylcholine was conducted in the following manner. Having collected all the necessary tissue in liquid  $N_2$  (described previously), each brain, or brain part, while still in the frozen state, was then rapidly weighed to the nearest fraction of a milligram on an electric, Mettler balance. Immediately after weighing, the tissue was placed in the extraction solution, kept in an ice bath to maintain its temperature near  $0^{\circ}C$ , and quickly hand-homogenized to assure that the frozen tissue did not thaw before dispersing into the solvent (Takahashi and Aprison, 1964). This, therefore, prevented subsequent destruction or synthesis of acetylcholine upon thawing (refer above; and MacIntosh and Perry, 1950). This sequence was repeated, until all of the tissue was processed. Then a thorough disintegration was carried out for 4 minutes, using a motor-driven homogenizer. The homogenate was heated in a boiling, water bath for 10 minutes and then allowed to cool. The centrifugate was transferred to another tube and the homogenizing tube was washed with 1 ml. of the Locke saline (without eserine) which was added to the rest of the homogenate. The supernatant was collected. The original homogenizing tube was washed once again but this time with distilled water; this washing was used to resuspend the precipitate from the centrifugation above. This suspension was centrifuged and the supernatant added to the first supernatant collected. At this point that the Ph of the extract was approximately 3-4, (pHydrion paper). In a few instances the extract appeared cloudy and could not be cleared by centrifugation.

However, following neutralization with N/3 NaOH (pHydrion paper), the cloudy elements were in all instances removed by centrifugation, resulting in a clear supernatant.

In experiments employing whole brain, the extract at this point was ready for assay and its volume was noted. However, when brain parts were used, an additional procedure was necessary. Brain tissue may contain substance P, as well as some other active polypeptides which are non-specific, smooth muscle stimulants (Amin, et al, 1954; Pernow, 1953; Takahashi and Aprison, 1964; and Toh, 1963 and 1967). If this material were present in the extracts at a certain concentration, it could interfere with the bioassay. Apparently whole brain levels of such material do not reach interfering proportions, but initial attempts to quantitate acetylcholine levels in brain parts proved difficult. Therefore, appropriate measures were taken to remove substance - P and other polypeptides from the extract. The neutralized, clear extract was incubated with 400 units/ml. solution of alpha-chymotrypsin (Sigma Chemicals) at 38° C for approximately 30 minutes. Treatment of the extract in this manner has been found to destroy substance - P and other polypeptides (Amin, et al, 1954; Aprison, et al, 1968; Pernow, 1953; Toh, 1963 and 1967; and Toru, et al, 1966), while having no effect on acetylcholine or on the assay of acetylcholine (Ryall, et al, 1964; and Takahashi and Aprison, 1964). This latter lack of effect on acetylcholine was verified on the ileum by this author. Incubation with alpha-chymotrypsin produced no precipitate or other observable changes in the clear extract (pH was checked and was not altered). After recording the final extract volume (of both

whole brain or brain parts), which averaged about 6 to 7 ml., approximately 2.5 to 3.0 ml. of each extract was separated, made alkaline by adding 0.4 ml. N/3 NaOH (pH 11) and heated in a boiling water bath for 5 minutes. This solution was allowed to cool, was centrifuged, and the clear supernatant collected and neutralized with N/3 HCl. Both the alkaline treated extract (S) and the original extract (U) were used in the subsequent bioassay. Treatment of part of the original tissue extract in this manner serves a dual purpose.

In 1944, Feldberg noticed that brain extracts contained substances which caused a sensitization of the assay tissue used to quantitate acetylcholine (Feldberg, 1944-45). He found that comparing the activity of these brain extracts with that of standard saline solutions of acetylcholine would result in erroneous determinations of brain acetylcholine concentrations. As a result, Feldberg designed a procedure to correct for this sensitization. It consisted simply in ridding a part of the brain extract of its acetylcholine by hydrolyzing it with a hot alkali. After neutralizing this solution, a known amount of acetylcholine was added; subsequently, this was used as the standard acetylcholine solution against which the untreated part of the brain extract was assayed (Feldberg, 1944-45). Since Feldberg's conception of this procedure, it has been used by investigators to control non-specific effects of tissue extracts on assay tissues and to assure accuracy and specificity of the bioassay (Bently and Shaw, 1952; Holmstedt, 1967; Kurokawa, et al, 1963; and Takahashi and Aprison, 1964).

Hot, alkali treatment serves the additional purpose of checking the extent to which the biological activity of the extract is due to



the presence of cholinomimetic substances. This is one of the more widely used criteria for identifying choline esters, since such treatment abolishes the biological activity of choline esters by hydrolyzing them (Aprison and Nathan, 1957-b; Banister, et al, 1953; Elliott, et al, 1950; Giarman and Pepeu, 1964; Hebb and Krnjevic, 1962; Hemsworth and Morris, 1964; Kovach, et al, 1957; Quastel, et al, 1936; Ryall, et al, 1964; and Whittaker and Wijesundra, 1952).

The biological activity of alkali - treated extracts was tested and shown to be virtually abolished, while the untreated extract still demonstrated very marked activity. Hot alkali treatment of the extract was the second of three criteria used here to identify the active principle in the extracts as a cholinomimetic substance.

While boiling in alkali is a standard procedure for the elimination of acetylcholine (Hebb and Krnjevic, 1962), it seemed possible to the author that other substances could also be destroyed in the process. Therefore, the effect of such treatment on serotonin and histamine (besides the active polypeptides, these are considered the major interfering factors (Toru, et al, 1966)) was tested in this thesis and the proposed destruction of acetylcholine was checked. When no alkali treatment was conducted, 100% of the acetylcholine added to the extracting medium was recovered in the absence of brain tissue (subsequent recovery experiments, performed by this author, in the presence of brain tissue determined that 71% of the added acetylcholine was recovered). Similarly, histamine and serotonin were recovered by approximately 100%. In similar experiments where hot alkali treatment was used, no acetylcholine was recovered, while only 68% of the serotonin

and only 13% of the histamine remained. These results are consistent with those of Amin, et al (1954) who found serotonin to be considerably inactivated by alkali treatment. Thus it was decided not to rely solely on this single criterion to identify the active principle as a choline ester and to use appropriate blockers in the bioassay, especially for serotonin and histamine.

Having therefore extracted the acetylcholine from the brain tissue, it remained yet to quantitate its concentration.

### C. ACETYLCHOLINE BIOASSAY

Since an adequate biochemical method for determining acetylcholine was unavailable, the use of a bioassay method for the quantitation of acetylcholine was decided upon. Of the many tissues available for the bioassay of acetylcholine, the terminal ileum of the guinea pig was chosen as the best preparation, since the ileum is highly sensitive to acetylcholine and has the added advantage of great rapidity and ease of operation, while still capable of providing accurate and reproducible results (Bentley and Shaw, 1952). The primary disadvantage is its responsiveness to substances other than acetylcholine. This drawback was minimized by the judicious use of select antagonists (Barlow and Khan, 1959; Kimura, et al, 1960; Toru, et al, 1966; and Turner, 1965).

An adult, male, colored guinea pig, weighing approximately 300-350 grams, was sacrificed by a blow on the back of the neck and the jugulars were severed. The terminal ileum was removed not including several centimeters proximal to the ileo-caecal junction) and

placed in an aerated beaker of Tyrode solution (consisting of 8.0 g. NaCl, 0.8 g. KCl, 0.8 g.  $\text{CaCl}_2$ , 4.0 g.  $\text{NaHCO}_3$ , 0.2 g.  $\text{NaH}_2\text{PO}_4$  and 4.0 g. glucose made up to 4 liters with distilled water). It was found that the sensitivity of the ileum could be improved if the Tyrode was kept at 32° C. Since only the most distal segments of this ileum were to be used for the assay, the unused, proximal end was marked with a piece of string.

After allowing the gut to stabilize in the beaker for a while, a small segment was removed (2-3 centimeters) and suspended in Tyrode solution at 32° C. in a 10 ml. isolated-tissue bath (a Palmer tissue bath) through which air was bubbled. The ileal segment was suspended from a Phipps and Bird Linear-Motion transducer which was cable-connected to a Texas Oscilloriter to record the isotonic, contractions of the gut by means of an heat stylus. The suspended tissue was again allowed to rest for about 30 minutes.

A constant 2 minute dose cycle was used in which an agonist was added once every 2 minutes to the tissue bath; a maximum contact time of 30 seconds was allowed before the drug was washed out with fresh warm Tyrode solution after which the gut was allowed to relax until the next dose. The different doses of drugs were obtained by varying the volume of the injected solution, usually 0.1 ml. or 0.2 ml. (Aprison and Nathan, 1957-b; and Bently and Shaw, 1952).

Aside from acetylcholine and related cholinomimetics, brain extracts contain materials that variably affect the guinea pig ileum (Barlow and Khan, 1959; Toh, 1963 and 1967; Toru, et al, 1966; and Turner, 1965). Several methods have been employed in this thesis to eliminate or

to account for them.

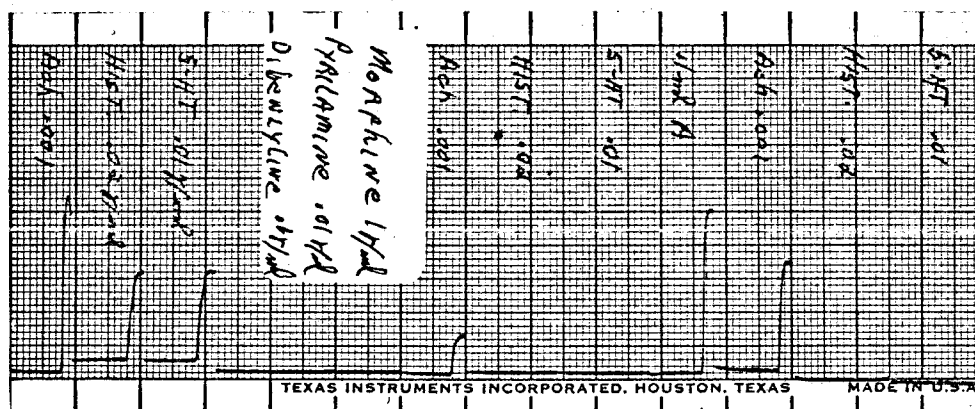
The uses of alpha-chymotrypsin, acid-heat, and Feldberg's alkali-treated-extract have already been explained. The presence of histamine and serotonin in brain tissue necessitates the use of appropriate antagonists for these substances during the bioassay (Aprison, et al, 1968; Ryall, et al, 1964; and Toru, et al, 1966). Extracts of whole brain acetylcholine were usually bioassayed in the presence of homochlorcyclizine (SA-97) and phenoxybenzamine hydrochloride (Dibenzyline, also a noted alpha sympatholytic agent) which were found to satisfactorily block both serotonin and histamine, (Kimura, et al, 1960; and Turner, 1965). Quantitation of brain part. acetylcholine was done in the presence of morphine sulfate and dibenzyline, serotonin antagonists, and pyrilamine maleate, an antihistaminic (Barlow and Khan, 1959; and Turner, 1965). Preliminary to every assay the efficacy of these antagonists was checked by testing adequate tissue responses to appropriate doses of acetylcholine chloride (0.001 gamma/ml. bath), histamine dihydrochloride (0.02 gamma/ml. bath) and serotonin (5-hydroxytryptamine creatinine sulfate) (0.01 gamma/ml. bath) in the presence and absence of the blockers. All the antagonists were added to the reservoir of Tyrode solution in appropriate amounts to result in the following bath concentrations: SA-97, 0.05 gamma/ml.; dibenzyline, 0.1 gamma/ml.; morphine, 1.0 gamma/ml.; and pyrilamine, 0.01 gamma/ml. (Kimura, et al, 1960; and Turner, 1965). While these drugs did abolish the responses to histamine and serotonin, they also depressed the response to acetylcholine. However, upon testing the effect of the brain extract, it was found that subsequent responses to acetylcholine, but not to serotonin or histamine, approached control levels. This is exemplified in

the recording in Figure 1. After a 20 minute contact with the gut of morphine, dibenzylamine and pyrilamine, the responses to histamine and serotonin are inhibited and the acetylcholine effect is depressed. But exposure to only one dose (0.1 ml.) of the A extract (code name for telencephalic extract) greatly improves the subsequent response to the same dose of acetylcholine but has no effect on histamine or serotonin.

A refinement used to account for interfering factors in the extract and to assure identity of the active principle in the extract involved the use of atropine sulfate. This will be discussed more fully below.

Having therefore eliminated the influences of histamine and serotonin, appropriate doses for both the untreated extract (the unknown or "U") and the alkali-treated extract (the standard or S), to which a known amount of acetylcholine HCl was added, were determined. The desired dose was one which caused an ileal contraction corresponding to the linear part of the dose-response curve for acetylcholine. The method of bioassay employed, called a 4-point or Latin square assay, entailed the use of a high ( $U_2$  and  $S_2$ ) and a low ( $U_1$  and  $S_1$ ) dose of both the unknown and standard solutions, respectively. It was important that the high and low doses of both the unknown and the standard be separated by the same logarithmic interval e.g. if  $U_2$  is twice the dose of  $U_1$ , this represents an arithmetic interval of 2 and a log interval of 0.301; therefore, whatever the dose of  $S_2$  is, it must be twice that of  $S_1$ , representing the same log interval of 0.301 (Bliss, 1952; Emmens, 1948; and Turner, 1965).

A Latin-square consists of 4 successive brackets of responses, each bracket being composed of a single response to  $U_2$ ,  $S_2$ ,  $U_1$  and  $S_1$ . Each of the 4 brackets contains a different sequence of these 4 responses.



**Figure 1.** Segment of record demonstrating the antagonism of serotonin (5-HT) (0.01 gamma/ml bath) and histamine (hist.) (0.02 gamma/ml bath) by morphine (1.0 gamma/ml bath), dibenzyline (0.1 gamma/ml bath) and pyrilamine (0.01 gamma/ml bath). Acetylcholine chloride (Ach) was given at a dose of 0.001 gamma/ml bath. 0.1 ml of the unknown, extract A (code name for telencephalic extract) was given. Subsequent tissue response to Ach is demonstrated, while histamine and serotonin are still blocked.

Figure 2 shows an exemplary 4-point assay used in this thesis.

Instead of using these response values directly for analysis and computation of acetylcholine concentration, they must first be corrected for the effect of non-specific smooth muscle stimulants which may be present in the extract. This corrective measure entails retesting the same  $U_2$ ,  $U_1$ ,  $S_2$  and  $S_1$  doses on the same strip of ileum but, this time, in the presence of atropine sulfate (0.02 gamma/ml. bath), an anticholinergic (Turner, 1965). The responses obtained in the presence of atropine are not due to acetylcholine and therefore are subtracted from the original bioassay response values for  $U_2$ ,  $S_2$ ,  $U_1$  and  $S_1$ . These corrected values were then subjected to statistical analysis and the relative potencies were determined to compute the concentration of acetylcholine in the brain tissue used according to Emmens (1948), see also (Bliss, 1952; Turner, 1965; and Wood, 1944). The cholinomimetic potencies of the extracts are expressed in terms of acetylcholine HCl standard.

This use of atropine represents the last of three recognized criteria used in this thesis for identifying the active constituent of the extract as being a cholinomimetic substance (Amin, et al, 1954; Aprison and Nathan, 1957; Banister, et al, 1953; Kovach, et al, 1957; Ryall, et al, 1964; and Sattin, 1966). The other two criteria described earlier were acid-heat stability and alkali-heat hydrolysis.

Furthermore, as mentioned earlier, atropine also allows an estimation of the interfering effects of non-specific smooth muscle stimulants.

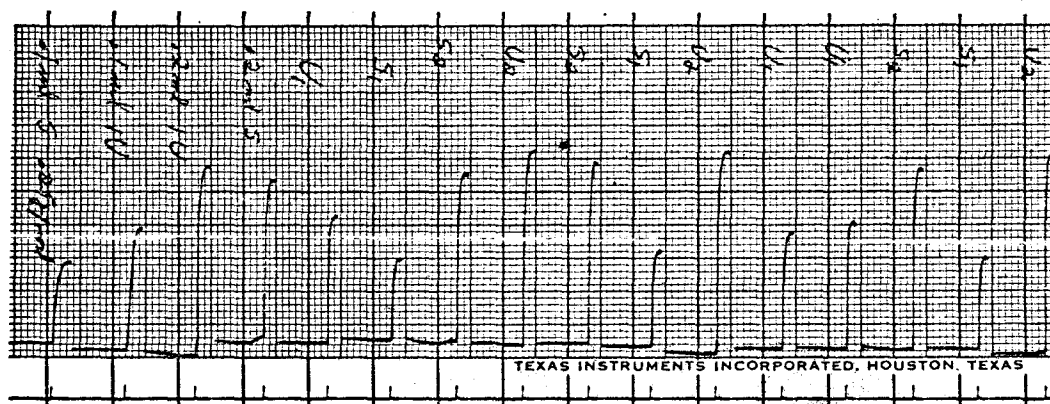


Figure 2. Segment of record demonstrating an exemplary 4-point Latin-square bioassay.  $S_1$  = low dose of standard (0.1 ml);  $S_2$  = high dose of standard (0.2 ml);  $U_1$  = low dose of unknown extract (0.1 ml);  $U_2$  = high dose of unknown extract (0.2 ml).  $S_1$  is equivalent to a 0.0025 gamma/ml dose of acetylcholine chloride and  $S_2$  is equivalent to a 0.005 gamma/ml dose.



## CHAPTER IV

### RESULTS

#### A. CONCENTRATIONS OF BRAIN ACETYLCHOLINE IN GENETICALLY DIFFERENT MICE

##### a. Intergeneric Whole Brain Acetylcholine Concentrations

Mean values of whole brain acetylcholine for each of the fourteen types of mice used are numerically depicted in Table 1. Included in this table are the standard error of each mean ( $\pm$  SE) and an indication of the number of experiments (and of the number of mice used per experiment) performed to obtain each mean value. It should be noted that the values for Onychomys leucogaster and Dipidomys deserti represent only pilot studies but were included to indicate their approximate relationship to the other mice. These data were expressed visually in Figure 3 to facilitate comprehension of the relationship of these mice types with regard to their whole brain acetylcholine. In this bar graph the mice genera were arranged along the abscissa in the order of increasing acetylcholine concentration. Acetylcholine values (gamma/gram) are given on the ordinate. The locations of the four strains of Peromyscus are accentuated by the hatched bars to readily contrast their acetylcholine levels with those of the four strains of Mus indicated by the speckled bars (this visual distinction will aid in subsequent discussion).

Proceeding from lowest to highest, the generic ranking order for whole brain acetylcholine concentration in adult, male mice is: Dipidomys deserti < Mus musculus C57 BL/6J < Microtus ochrogaster < Mus musculus SC1 < Mus musculus "Missouri" < Mus musculus CF1 < Peromyscus maniculatus "Colorado" < Dipidomys meriami < Peromyscus maniculatus gracilis < Reithrodontomys raviventris < Perognathus longimembris Bailli < Peromyscus

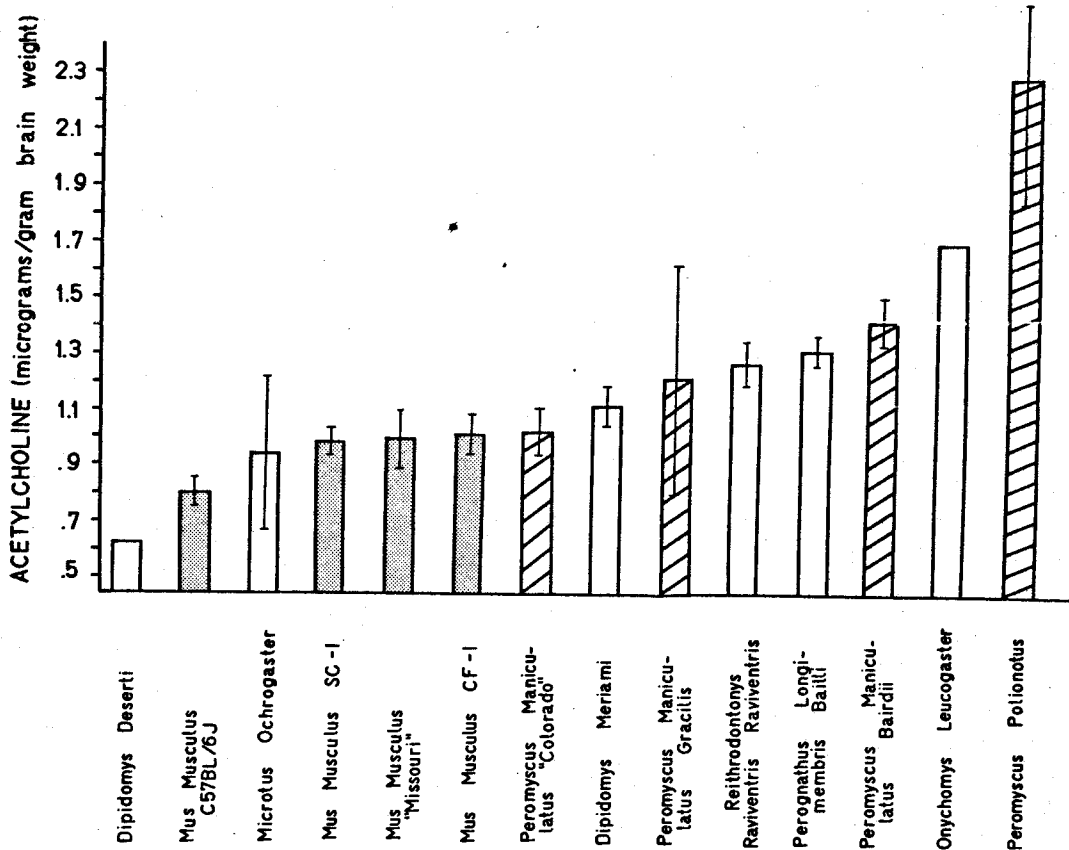
Type Of Mice	Body Weight, Grams $\pm$ SE	Whole Brain Weight, Grams $\pm$ SE	Ach, Gamma/Gram $\pm$ SE
Mus musculus C57Bl/6J	27 $\pm$ 1.3 (17)*	0.43 $\pm$ .01 (17)*	0.75 $\pm$ .09 (15/5)**
Mus musculus CF-1	35 $\pm$ 1.3 (18)	0.43 $\pm$ .01 (18)	1.05 $\pm$ .07 (15/5)
Mus musculus "Missouri"	15 $\pm$ 1.6 (6)	0.38 $\pm$ .01 (6)	1.00 $\pm$ .11 (6/2)
Mus musculus SC-1	37 $\pm$ 1.2 (104)	0.48 $\pm$ .01 (61)	0.96 $\pm$ .05 (12/4)
Microtus ochrogaster	48 $\pm$ 2.0 (11)	0.61 $\pm$ .01 (11)	0.95 $\pm$ .28 (11/4)
Onychomys leucogaster***	36 $\pm$ 0 (2)	0.61 $\pm$ 0 (2)	1.71 (2/1)
Peromyscus maniculatus Bairdii	18 $\pm$ 2.1 (8)	0.48 $\pm$ .01 (8)	1.41 $\pm$ .09 (8/3)
Peromyscus maniculatus "Colorado"	19 $\pm$ 1.1 (9)	0.56 $\pm$ .01 (9)	1.06 $\pm$ .09 (9/3)
Peromyscus maniculatus Gracilis	20 $\pm$ 1.1 (9)	0.65 $\pm$ .02 (9)	1.22 $\pm$ .40 (9/3)
Peromyscus polionotus	14 $\pm$ .6 (11)	0.48 $\pm$ .00 (11)	2.30 $\pm$ .44 (8/3)
Reithrodontomys raviventris Raviventris	17 $\pm$ .9 (4)	0.43 $\pm$ .00 (4)	1.29 $\pm$ .08 (4/2)
Dipodomys meriami	39 $\pm$ 2.6 (2)	1.37 $\pm$ .28 (2)	1.13 $\pm$ .07 (2/2)
Dipodomys deserti***	146 (1)	1.69 (1)	0.63 (1/1)
Perognathus longimembris Bailli	35 $\pm$ 3.3 (8)	0.66 $\pm$ .03 (8)	1.33 $\pm$ .05 (6/3)

Table 1. Whole brain acetylcholine (Ach) concentrations (mean  $\pm$  SE, micrograms/gram), brain weights (mean  $\pm$  SE, grams) and body weights (mean  $\pm$  SE, grams) of genetically distinct adult, male mice.

\* Total number of mice used per mean value

\*\* Total number of mice used/number of determinations

\*\*\* Pilot study



**Figure 3.** Whole brain acetylcholine concentrations (ordinate — micrograms/gram  $\pm$  SE) of 14 genetic varieties of adult, male mice (abscissa — arranged from left to right in order of increasing Ach concentrations). The speckled bars distinguish the Mus genus from the Peromyscus genus, hatched bars.

maniculatus Bairii < Onychomys leucogaster < Peromyscus polionotus.

As can be seen from figure 3, there is a considerable degree of overlapping but certain outstanding differences are suggested. Intergeneric (between genera) differences exist in whole brain levels of acetylcholine, a well established fact in the literature (Bennett, et al, 1960). However, this demonstration in mice and, especially, in the genera used here is unique. An approximately 202% difference was found between the genera with the highest and lowest acetylcholine values; Peromyscus polionotus contained the highest and Dipodomys deserti the lowest values of all the mice studied. Not only intergeneric and intrageneric (between species of the same genus), but also interstrain (between strains of the same genus and species) differences were evident.

Intragenerically, Dipodomys meriami contained almost twice as much acetylcholine on a per gram basis as Dipodomys deserti. Also, there was from an 63% to a 117% difference in acetylcholine concentration between Peromyscus polionotus and the three Peromyscus maniculatus strains, the former species surpassing the latter. While interstrain dissimilarities were also apparent between the four Mus musculus strains and between the three Peromyscus maniculatus strains, these differences never exceeded 40% in Mus and 33% in Peromyscus.

The mean acetylcholine levels for all four types of Peromyscus (hatched bars) (figure 3) and all four strains of Mus musculus (speckled bars) reveals a distinct tendency for Peromyscus to contain more acetylcholine per gram of brain tissue than Mus. In fact, with the possible exception of Microtus and Dipodomys deserti, all of the genera investigated here surpassed Mus in whole brain acetylcholine concentration.

b. Acetylcholine In Brain Parts Of Four Strains Of *Mus musculus*

During the course of this work data on acetylcholine levels in brain parts of four strains of *Mus musculus* (C57BL/6J, CF1, SC1, and ICR) was obtained. The brain parts investigated were telencephalon (T) (cerebral cortex, hippocampus, basal ganglia and olfactory bulbs); midbrain-diencephalon (M-D) (thalamus, hypothalamus and midbrain); and pons-medulla (P-M). Bioassay of cerebellar (C) extracts was prevented by the low levels of acetylcholine and the presence of an interfering factor.

The data in table 2-A shows that mice strains can be characterized by the regional levels and relative distributions of acetylcholine within their brains. Acetylcholine concentrations are expressed as mean  $\pm$  S.E., micrograms/gram; also included is an indication of the number of experiments carried out to obtain the mean value and the number of mice used per experiment. The C57BL/6J strain of *Mus musculus* has the lowest T acetylcholine of the four strains studied. It is second highest, however, in both M-D and P-M acetylcholine concentrations. In this strain both the M-D and P-M contain approximately equivalent concentrations of acetylcholine, while the T has 170% less acetylcholine than either of the two brain parts.

The level of T acetylcholine in CF1 ranks second highest among the four strains; its M-D and P-M concentrations were also second highest. Within brain comparison in this strain ranks T acetylcholine about 32% lower than M-D levels and 23% lower than P-M and M-D barely surpasses P-M acetylcholine concentration by about 14%.

The T acetylcholine of the SC1 strain is relatively equivalent to that of CF1 mice, ranking them both second highest of the four strains.

A. Regional Brain Acetylcholine Concentrations  
(mean  $\pm$  SE, micrograms/gram)

Strains of <u>Mus musculus</u>	T <sup>+</sup>	M-D <sup>++</sup>	C <sup>+++</sup>	P-M <sup>++++</sup>
C57Bl/6J	0.60 $\pm$ .10 (8/2)*	1.60 $\pm$ .18 (8/2)	-	1.58 (4/1)
CF1	0.82 $\pm$ .07 (35/7)	1.21 $\pm$ .08 (35/7)	-	1.06 $\pm$ .10 (35/7)
SC1	0.84 $\pm$ .05 (43/9)	1.09 $\pm$ .03 (39/8)	-	0.83 $\pm$ .05 (35/7)
ICR	1.36 $\pm$ .20 (20/4)	1.98 $\pm$ .15 (15/3)	-	2.10 $\pm$ .60 (15/3)

B. Brain Part Weights (mean  $\pm$  SE, milligrams)

Strains of <u>Mus musculus</u>	T	M-D	C	PM
C57Bl/6J	251.8 $\pm$ 2 (12)**	73.4 $\pm$ 1 (12)	45.2 $\pm$ 1 (12)	52.6 $\pm$ 2 (12)
CF1	255.6 $\pm$ 5 (38)	81.7 $\pm$ 1 (38)	55.1 $\pm$ 1 (38)	60.7 $\pm$ 1 (38)
SC1	285.3 $\pm$ 4 (43)	89.7 $\pm$ 1 (43)	-	72.2 $\pm$ 1 (43)
ICR	250.0 $\pm$ 7 (30)	87.9 $\pm$ 3 (30)	54.8 $\pm$ 1 (30)	59.6 $\pm$ 2 (30)

Table 2. A. Acetylcholine concentrations (mean  $\pm$  SE, micrograms/gram) and B. average weights (mean  $\pm$  SE, milligrams) of brain parts of four strains of adult, male Mus musculus mice.

+ Telencephalon  
++ Midbrain-Diencephalon  
+++ Cerebellum  
++++ Pons-Medulla

\* Total number of mice used/number of determinations  
\*\* Total number of mice used per mean value

Unique to SC1 is the fact that its acetylcholine concentration of the P-M is the lowest for this brain part of all four strains; furthermore, SC1 ranks lowest in acetylcholine levels of M-D. Within brain comparison of this strain of Mus musculus is characterized by equal acetylcholine concentrations of T and P-M. Both of these regions are 23% lower in acetylcholine than the M-D.

Finally, ICR mice are distinguished by having the highest acetylcholine concentrations in all three brain regions. M-D and P-M are approximately equal in acetylcholine concentrations, surpassing that of the T by about 50%.

## B. COMPARATIVE BRAIN WEIGHTS

### a. Intergeneric Whole Brain Weights

As with the acetylcholine data, the whole brain weights, numerically expressed in Table 1, are graphically illustrated in Figure 4 to facilitate comparisons between mice types. The special designation of Peromyscus (hatched bars) and Mus (speckled bars) was employed again. The mice genera on the abscissa are arranged in the order of increasing acetylcholine concentrations, the ordinate designating the brain weight in grams.

In terms of whole brain weight Mus musculus "Missouri" has the lightest brain followed by Mus musculus C57BL/6J and CF1 and Reithrodontomys raviventris Raviventris which three mice types have brains of approximately equal weight. The next heaviest brains are found in Mus musculus SC1, Peromyscus maniculatus Bairdii and Peromyscus polionotus, all of which have equally weighted brains. These are followed in order of increasing whole brain weight by Peromyscus maniculatus "Colorado", Microtus ochrogaster and Onychomys leucogaster, Peromyscus maniculatus Gracilis, Perognathus

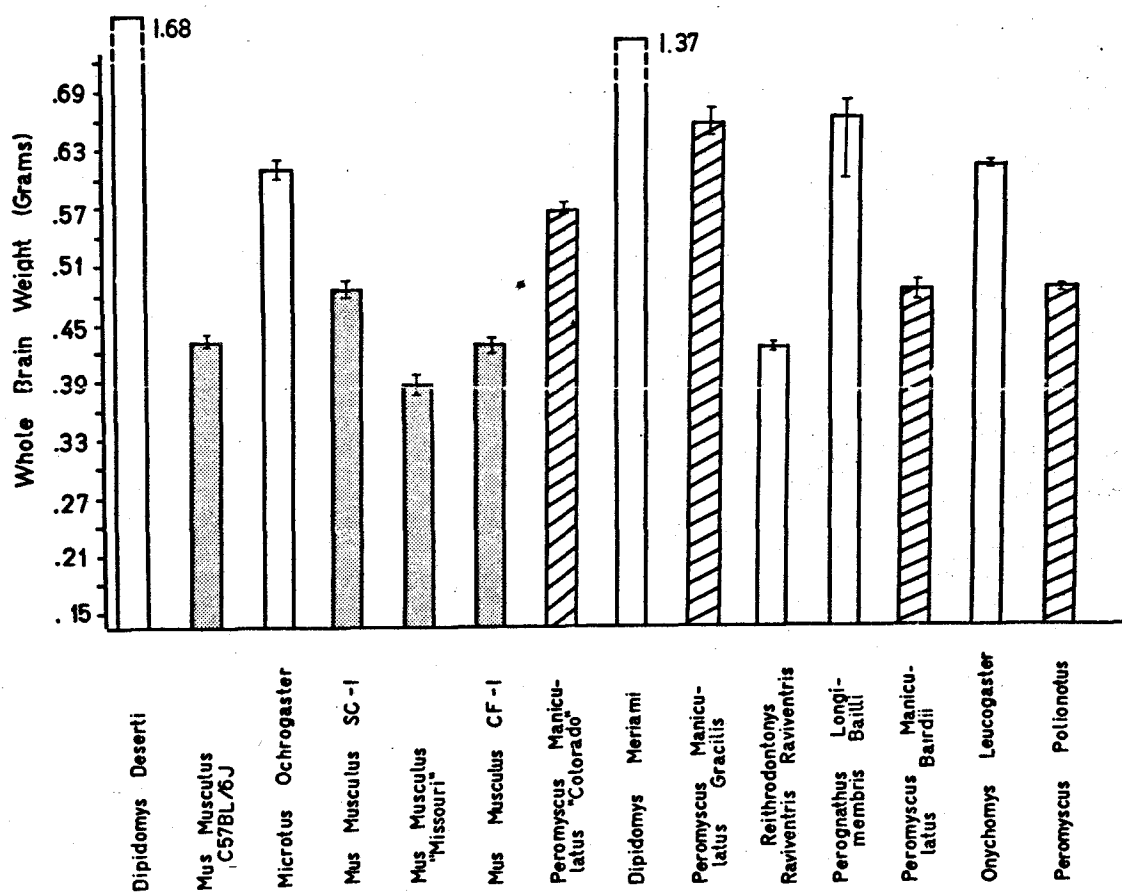


Figure 4. Whole brain weights (ordinate — grams  $\pm$  SE) of 14 genetic varieties of adult, male mice (abscissa — arranged from left to right in order of increasing Ach concentrations). The speckled bars distinguish the *Mus* genus from the *Peromyscus* genus, hatched bars.



longimembris Bailli, Dipodomys meriami, and Dipodomys deserti.

With the exception of the Dipodomys genus, which was found to have a brain many times heavier than any of the other genera studied, intergeneric (between different genera) differences in brain weight, though present, were not as marked as were the whole brain acetylcholine differences. The greatest difference in brain weight, excluding Dipodomys, was between Perognathus and Mus musculus "Missouri", the former being 74% heavier than the Mus. Peromyscus (hatched bars in Figure 4) tended to have heavier brains than Mus (speckled bars). Furthermore the Mus genus had brains lighter than most of the mice genera used in this study with the exception of Reithrodontomys.

Intragenetic (between species of the same genus) differences, though small, were also present. The brains of Peromyscus polionotus mice were slightly (from 0% to 23%) lighter than three Peromyscus maniculatus strains, while Dipodomys deserti had a 23% heavier brain than Dipodomys meriami.

The interstrain brain differences were also small, the highest differences being only 35% for Peromyscus maniculatus strains and 26% for the Mus musculus variety.

#### b. Brain Part Weights Of Four Strains Of Mus musculus

The mean weights in milligrams of the telencephalon (T), midbrain-diencephalon (M-D), cerebellum (C) and pons-medulla (P-M) of Mus musculus C57BL/6J, CF1, SCL and ICR mice are found in Table 2-B along with standard errors (+SE) and an indication of the number of mice used per average value.

In all four strains of Mus musculus the proportionate weight of each part relative to the weight of the whole brain is characteristically the same. The T region comprises approximately 57% of the entire brain;

the M-D composed 18%; the C 11%; and P-M 13%. There are, however, differences between weights of similar brain parts from strain to strain. The SC1 strain has the heaviest T region as well as the heaviest P-M of the four strains. The other three strains have equi-weighted T regions. Mus musculus SC1 and ICR contain the heaviest M-D brain parts, followed by CF1 and C57BL/6J. This latter strain ranks lowest in comparative weights of C and P-M sections. The C weights of CF1 and ICR are equivalent (no data was obtained on C weight of SC1) as are their P-M weights.

#### C. COMPARATIVE BODY WEIGHTS OF GENETICALLY DIFFERENT MICE

Table 1 contains the numerical averages, the standard errors ( $\pm$  SE) and the number of mice used to obtain each mean of the body weights of 7 genera, 9 species and 14 strains of adult, male mice. These values are visually depicted in Figure 5, the genera of mice being arranged along the abscissa in the order of increasing acetylcholine concentrations and the body weight in grams being shown on the ordinate. The hatched bars are again used to distinguish the Peromyscus genus from the Mus genus, represented by the speckled bars.

In the order of increasing body weight the mice are ranked as follows: Peromyscus polionotus < Mus musculus "Missouri" < Reithrodontomys raviventris < Raviventris < Peromyscus maniculatus Bairdii < Peromyscus maniculatus "Colorado" < Peromyscus maniculatus Gracilis < Mus musculus C57BL/6J < Mus musculus CF1 and Perognathus longimembris Bailli < Onychomys leucogaster < Mus musculus SC1 < Dipodomys meriami < Microtus ochrogaster < Dipodomys deserti.

There are several salient features of this body weight data. The genus closest to Dipodomys deserti in body weight is Microtus which is still

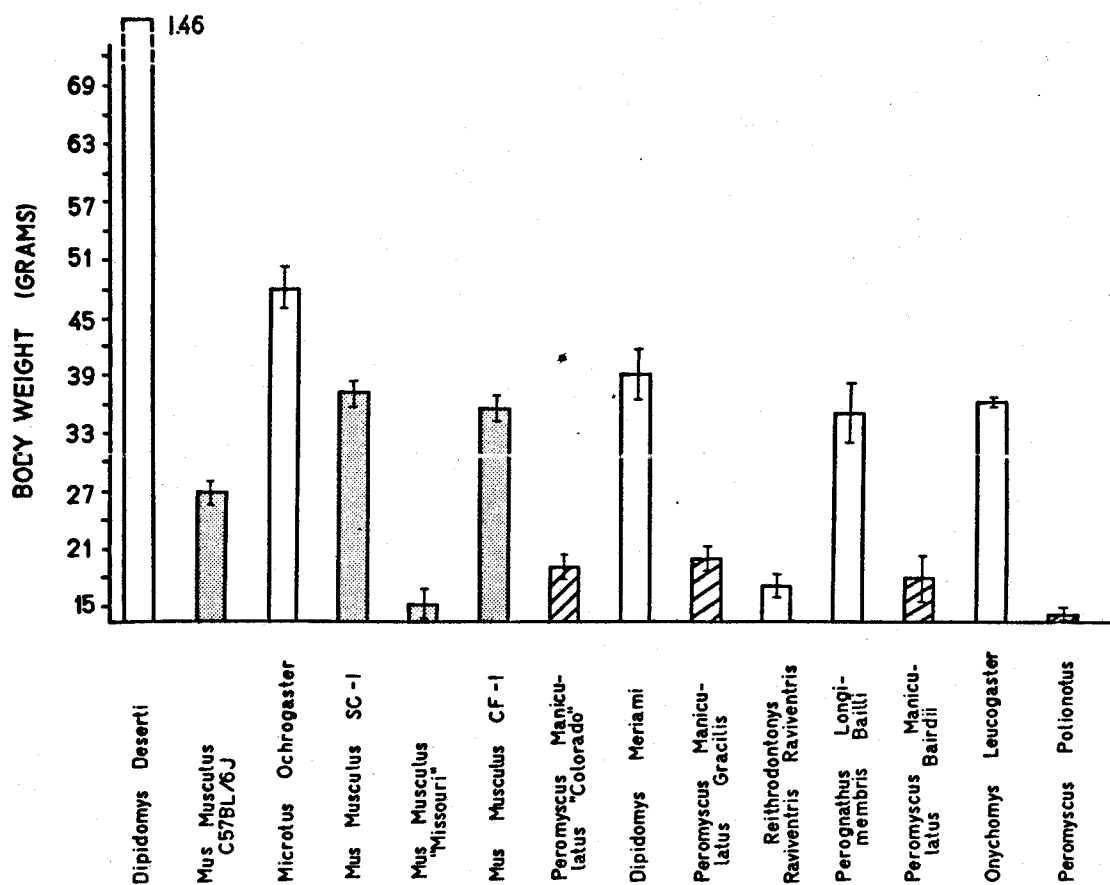


Figure 5. Body weights (ordinate — grams  $\pm$  SE) of 14 genetically distinct adult, male mice (abscissa — arranged from left to right in order of increasing Ach concentrations). The speckled bars denote the *Mus* genus and the hatched bars *Peromyscus*.

more than 67% lighter than the Dipodomys deserti. Dipodomys meriami is more than 74% lighter in body weight than Dipodomys deserti although a comparison of their brain weights shows no such diversity.

Another outstanding, intergeneric difference is between Peromyscus and Mus. The Mus genus (with the exception of Mus musculus "Missouri") far outweighs the Peromyscus genus. Furthermore, Peromyscus is outweighed by every genus investigated here except Reithrodontomys.

The greatest interstrain difference is between Mus musculus "Missouri" and the other three strains of Mus. The "Missouri" strain ranges from 44% to 60% lighter in body weight than the other three Mus musculus strains. In this respect the "Missouri" mouse resembles the Peromyscus and Reithrodontomys genera more than the Mus genus.

## CHAPTER V

### DISCUSSION OF INTERGENERIC WHOLE BRAIN LEVELS OF ACETYLCHOLINE IN SEVEN GENERA AND FOURTEEN STRAINS OF MICE

Neurochemical parameters other than acetylcholine have been investigated at this laboratory employing most of the same mice types used in this thesis. Karczmar, et al, (1968) reported on the whole brain levels of AChE and total ChE, and Richardson (unpublished data) has investigated the brain concentrations of catecholamines and serotonin (5-HT). With the authors' permission their data is reproduced here to allow comparison with acetylcholine levels (Figures 6, 7 and 8).

#### A. GENETIC RELATIONSHIP OF WHOLE BRAIN ACETYLCHOLINE TO TOTAL CHOLINESTERASE AND ACETYLCHOLINESTERASE ACTIVITIES

In Figure 6 whole brain concentrations of acetylcholine (abscissa) of 7 genera, 8 species and 12 strains of mice were plotted against brain total ChE activity (left ordinate) and AChE activity (right ordinate) of similar mice. Acetylcholine values were those reported in this paper and the cholinesterase values were taken from the work of Karczmar, et al, (1968). Superimposing these two curves is a means of establishing a visible correlation between the parameters on the right and left ordinates. However, there is no obvious general tendency for either of the curves to form a directly or indirectly related function to the acetylcholine values on the abscissa. This, therefore, indicates the lack of an intergeneric relationship between acetylcholine and cholinesterase activity.

Intragenerically, however, there does appear a tendency for acetylcholine concentrations to be directly related to cholinesterase activity. This tendency can be seen within both the Mus and Peromyscus genera. Mus musculus

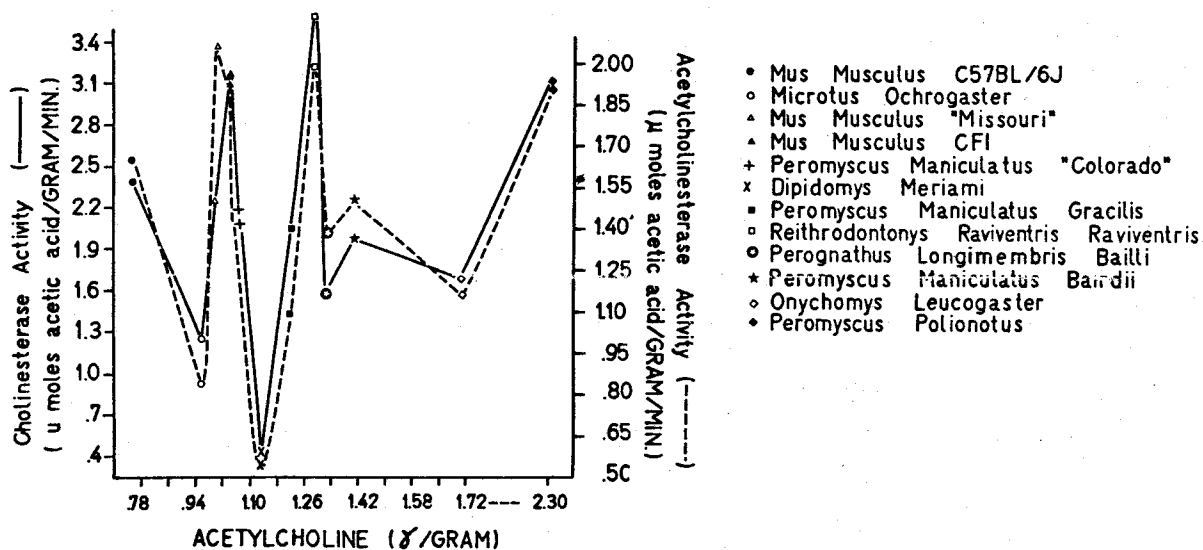


Figure 6. Intergeneric comparison of whole brain acetylcholine concentrations (abscissa — micrograms/gram), total brain cholinesterase activity (left ordinate — micromoles acetic acid/gram/minute) and acetylcholinesterase activity (right ordinate — micromoles acetic acid/gram/minute). Total cholinesterase and acetylcholinesterase activities were taken from Karczmar, et al, (1968) with the authors' permission.

"Missouri" and CF1 contain approximately equivalent acetylcholine concentrations both, therefore, surpassing Mus musculus C57BL/6J. This same relationship is demonstrated for cholinesterase activity. Peromyscus polionotus contains the highest acetylcholine concentration as well as cholinesterase activity of its genus. It is followed by Peromyscus maniculatus Bairdii with lower amounts of both substances and, finally, Peromyscus maniculatus Gracilis and "Colorado" which are lowest in both acetylcholine and cholinesterase.

Since acetylcholine levels are known to fluctuate with activity (Aprison, et al, 1968), their measurement in different genera and species of mice gives no indication as to the genetic control of its synthesis. Also, since cholinesterase is measured in terms of activity, there is no way to deduce the actual, genetically determined amount of enzyme present in the brain tissue. Therefore, the present data cannot ascertain whether or not these acetylcholine and cholinesterase values are genetically dependent or fortuitously correlated. In this regard Bennett, et al, (1960), Roderick, (1960) and Rosenzweig, et al, (1960), using specific breeding techniques, have suggested that these substances are, in fact, genetically independent. However, these data do show a direct relationship (not necessarily a genetic dependency) between acetylcholine concentration and cholinesterase activity intragenerically. A direct relationship has been previously suggested to exist between acetylcholine concentrations and cholinesterase activity (Burkhalter, et al, 1957; and Rosenzweig, et al, 1960). These investigators have reported that an increased acetylcholine concentration will lead to a compensatory increase in cholinesterase activity. It is interesting to note at this point that a very good positive correlation was found between brain

cholinesterase activity and production of gamma-aminobutyric acid (GABA) in 5 different strains of rats (Rick, et al, 1968). Furthermore, high brain levels of GABA in seizure-prone strains of mice were positively correlated with high acetylcholine levels (Naruse, et al, 1960). This was interpreted by Roberts (1960 and 1966) to be a compensated increase in GABA in response to the high acetylcholine, similar to the case with cholinesterase activity. Such a compensatory mechanism could serve to explain the tendency for cholinesterase activity to vary directly with acetylcholine levels in the Mus mice and in the Peromyscus mice. However, if this is true, then it must be concluded that this compensatory phenomenon does not occur between animals more distantly related than different species, since acetylcholine and cholinesterase do not relate intergenerically in this study.

B. GENETIC RELATIONSHIP OF WHOLE BRAIN ACETYLCHOLINE CONCENTRATIONS TO BRAIN SEROTONIN AND BIOGENIC AMINES

Although there is a lack of an intergeneric correlation with cholinesterase, a comparison of acetylcholine concentrations with whole brain levels of norepinephrine (NE) and 5-HT (Richardson, unpublished data) revealed a surprisingly definite direct relationship (Figure 7). The direct correlation between acetylcholine (values on abscissa), 5-HT (left ordinate) and NE (right ordinate) is evident by the slanting of both curves to the right. The similarity between the two curves indicates direct relationship between NE and 5-HT, as well. Unlike cholinesterase activities, NE and 5-HT correlate with acetylcholine better on an intergeneric basis than on an intrageneric level. The apparent intergeneric relationship of acetylcholine, NE and 5-HT may represent a state of interaction between these neurochemical systems that is basic and generally operative in all of these mice. The



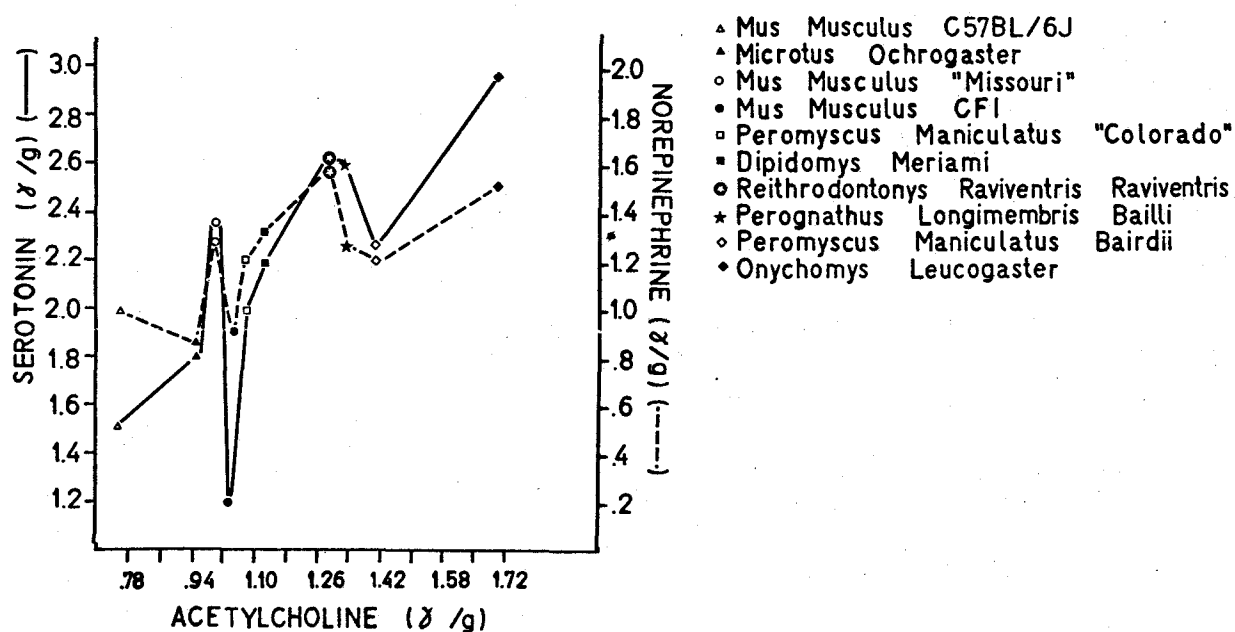


Figure 7. Intergeneric comparison of whole brain acetylcholine concentrations (abscissa — micrograms/gram), serotonin concentrations (left ordinate — micrograms/gram) and norepinephrine concentrations (right ordinate — micrograms/gram). Values for whole brain serotonin and norepinephrine concentrations were taken from Richardson (unpublished data) with the investigator's permission.

level of activity of this state of interaction or balance, indicated by the concentration of each of these substances, may determine the behavioral and/or physiological uniqueness of each genus of mouse. The intragenetic variations of the NE and 5-HT levels relative to the acetylcholine levels may represent biochemical fluctuations which are an expression of the uniqueness of one species compared to another.

The existence of interacting central neurochemical systems was suggested by Hess (1954). This suggestion was adapted by Brodie, et al, (1959) to include the cholinergic and the adrenergic systems. These systems were postulated to regulate behavior by operating by a continuous balance between them. Carlton (1963) offered evidence which supported the idea that within this balanced system, acetylcholine functions to control, by appropriate depression or inhibition, the excitatory activities of the adrenergic system. Other investigators have supported this theory, for example Mennear (1965) presented evidence showing that cholinomimetics depress spontaneous, exploratory, motor activity while amphetamine stimulates such activity. Depression of the antagonistic cholinergic system with scopolamine, increased exploratory behavior in a manner similar to amphetamine. The intergeneric data presented here adds additional support to the theory of a basic adrenergic-cholinergic balance, since these biochemical systems appear to compliment each other across several different genera of mice. However, this direct relationship of acetylcholine with the adrenergic system appears to hold primarily for NE and not for dopamine or DOPA, both precursors of NE. Examination of Figure 8, which is a graphic comparison of whole brain acetylcholine concentrations (abscissa) to dopamine (left ordinate) and DOPA (right ordinate) concentrations in the same mice

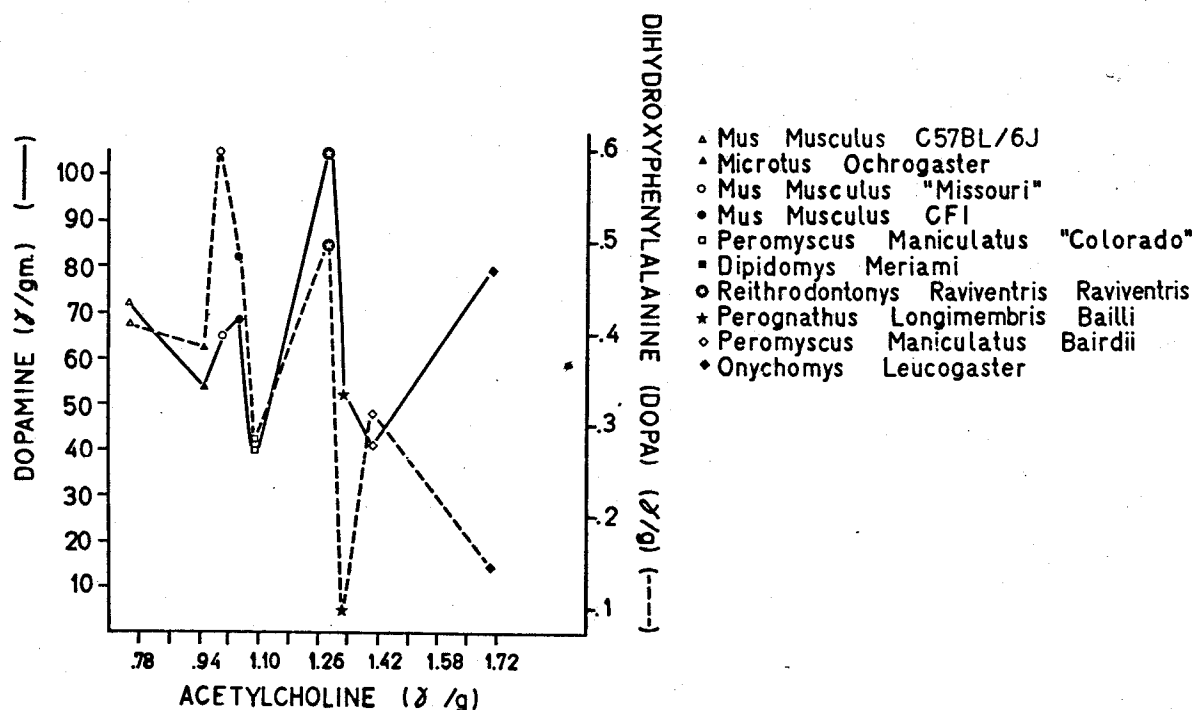


Figure 8. Intergeneric comparison of whole brain acetylcholine concentrations (abscissa — micrograms/gram), dihydroxyphenylethylamine (DOPAMINE) (left ordinate — micrograms/gram) and dihydroxyphenylalanine (DOPA) (right ordinate — micrograms/gram). DOPAMINE and DOPA concentrations were taken from Richardson (unpublished data) with the investigator's permission.

used above, shows no clear relationship between acetylcholine and either DOPA or dopamine.

However, the present data dose show that in addition to NE, acetylcholine was similarly related to 5-HT, thereby involving another neurochemical system. The finding of this relationship across several genera of mice supports a theory proposed by Aprison (1962). While accepting the proposition that an adrenergic-cholinergic balance could effect "coarse" control over behavior, he questioned the ability of such a system to produce "fine" behavioral control. Rather, he proposed that the serotonergic system exerts a subtle control or modulating influence on the main cholinergic system to effect such "fine" behavioral control. Aprison presented evidence that cholinesterase activity can be inhibited by high amounts of 5-HT, thereby facilitating cholinergic activity. He went on to suggest that lower amounts of 5-HT effect a negative chemical feedback by inhibiting cholinergic receptors. Even though the data presented in Figure 7 does not enable an appraisal to be made by this author of the mechanism proposed by Aprison, it is consistent with his theory of a modulator role for 5-HT and his subsequent finding of a linear relationship between acetylcholine and 5-HT, as well as NE, in different brain regions (Aprison and Takahashi, 1965).

C. INTERGENERIC RELATIONSHIP OF WHOLE BRAIN ACETYLCHOLINE AND SEVERAL BEHAVIORAL AND PHYSIOLOGICAL PARAMETERS

While a relationship is indicated between acetylcholine, NE and 5-HT, it is not within the scope of this thesis to delineate the direction of interaction, i.e., which system antagonizes or facilitates the other systems, or the degree of activity of each neurochemical system that is required to produce a specific behavior. Rather, interpretation of the data

presented here will be limited to describing the general involvement of the cholinergic system in an array of behavioral and physiological parameters previously reported from this laboratory using several of the same genera of mice used here (Karczmar and Scudder, 1967). On the basis of their acetylcholine concentrations the mice can be divided into two major groups: 1) the mice representing low acetylcholine concentrations consisted primarily of the 3 strains of Mus musculus, C57BL/6J, "Missouri" and CF1; the low mean brain acetylcholine of the Microtus places this animal in the same category with Mus mice; 2) high brain acetylcholine was found in the Peromyscus, Perognathus, Onychomys and Reithrodontomys genera. Since the clearest behavioral differences were exemplified between Mus and Peromyscus mice, these 2 genera were distinguished in the bar graphs (Figure 3) to aid comparison of Mus, representing the low acetylcholine group, and Peromyscus, representing the high acetylcholine group.

#### a. Exploratory Behavior

Behaviorally, Mus mice are characterized by a higher amount of exploratory behavior than Onychomys, Peromyscus or Microtus (Scudder, et al, 1969; and Karczmar and Scudder, 1968).

Contrasting Mus with Onychomys and Peromyscus indicates an inverse relationship between exploration and cholinergic function. This receives support from the finding that scopolamine, a cholinolytic, increases spontaneous motor activity (exploratory behavior) (Mennear, 1965; Parkes, 1965; and Meyers and Domino, 1964). Furthermore, Mennear (1965) also showed that tremorine, a cholinomimetic, depresses spontaneous exploratory behavior, thereby substantiating the finding here that higher acetylcholine levels are associated with lowered exploration. However, since the low exploration of

Microtus is related to a level of acetylcholine even lower than that of Mus, as a genus, this seems to indicate that an optimum level of the cholinergic system exists which results in maximum exploratory behavior (based on the theory of "optimum levels" by Malmö, 1966). This optimizing is embodied in the Mus genus. Any deviation in terms of acetylcholine levels would result in decreased exploratory behavior; higher acetylcholine, as in Peromyscus and Onychomys, or lower acetylcholine, as in Microtus, are both associated with exploratory behavior lower than that exhibited by Mus. On the other hand, the factor of chance occurrence of either low acetylcholine or low exploratory behavior in Microtus must also be considered as a possibility. If this were the case, then a "optimum" theory for exploration and whole brain acetylcholine could not be evoked. Rather, an inverse relationship between these two parameters would apply, exploratory behavior increasing as brain acetylcholine concentration decreases.

b. Aggression

In a similar fashion Mus was found to be more aggressive than mice higher in acetylcholine, Onychomys and Peromyscus, as well as lower in acetylcholine, Microtus (Scudder, et al, 1969; and Karczmar and Scudder, 1968). Since these determinations of aggression were based partly on isolation induced aggression, it is significant to the findings presented here that Geller, et al, (1965) and La Torre (1968) found greater cholinesterase activity, indicative of high acetylcholine levels (Burkhalter, et al, 1957), in the aggregated (non-aggressive) than in the isolated (aggressive) animals. Therefore, aggressiveness can be associated with a certain degree of cholinergic inactivation, since low acetylcholine Mus are more aggressive than high acetylcholine Peromyscus or Onychomys. However,

since low aggressiveness is found in mice which are also low in acetylcholine, Microtus, it seems that too much cholinergic inactivity is also detrimental to aggressive behavior. This indication receives support from the fact that scopolamine, a cholinolytic, is one of the most potent antiaggressive agents known (Janssen, et al, 1960). Therefore, very high acetylcholine levels as well as very low levels depress aggressiveness; but maximum aggression can be obtained by an optimum of the cholinergic system between the two extremes (based on "optimum level" theory of Malmo, 1966).

As with exploratory behavior, the application of the theory of "optimum levels" to acetylcholine and aggression may be rather speculative. The low acetylcholine levels of Microtus may have occurred by chance and may be unrelated to aggressiveness. In this event aggressiveness would be inversely related to whole brain acetylcholine. Nevertheless, the antiaggressive effectiveness of scopolamine would still imply a relatedness between depressed aggression and very low levels of cholinergic functioning. Undoubtedly, a more reasonable explanation for these findings could be arrived at, if more specific brain parts were investigated, since whole brain studies may mask subtle regional changes.

#### c. Periodic Motor Immobility ("Freezing") And Stereotypy

According to earlier reports, mice exhibiting relatively greater exploratory and aggressive behavior, such as Mus, demonstrate a much lower degree of "emotionality" in the form of freezing and stereotypy (Bovet, et al, 1969; Karczmar and Scudder, 1967; Maas, 1962 and 1963; Scudder, et al, 1967; and Sudak and Maas, 1964) than do such genera as Onychomys and Peromyscus which are less exploratory and aggressive and more prone to stereotypic and freezing behaviors (Scudder, et al, 1969). Implicated, therefore, with high

"emotionality," as exemplified by freezing and stereotypy, is a high brain level of acetylcholine. Contrariwise, lowered cholinergic functioning is associated with a depression of freezing and stereotypy. The association of high cholinergic activity with freezing or immobility was also implied by Zetler (1968) who found that various central cholinergic stimulants can induce a state of tonic immobility (catatonia). A similar state of immobility induced by select brain lesions (Delini-Stula, 1968) can be alleviated by anticholinergic drugs (Zetler, 1968). Furthermore, Aprison and Nathan (1957-a) implicated increased cholinergic functioning in compulsive, stereotypic motor behavior. Additional support for an hyperactivity of the cholinergic system in high "emotionality" was provided by Ruther, et al (1966). They attributed the reduction by imipramine of an "emotional" reaction to stress as being due to its anticholinergic effects. Therefore, the association of high brain acetylcholine with high "emotionality," and low acetylcholine with low "emotionality" seems well-supported in literature.

#### d. Motor Activity

The excessive daytime total motor activity of Microtus and Mus clearly distinguished them from Peromyscus, Perognathus and Onychomys mice (Karczmar and Scudder, 1967). Accordingly, therefore, low brain acetylcholine accompanies high total motor activity and high acetylcholine is associated with low motor activity. This inverse relationship between acetylcholine and motor activity, however, conflicts with the reports of other investigators. Anden, et al, (1966) considers muscular tremor and rigidity to be hypermotor symptoms of an uninhibited central cholinergic motor system. Their results are supported by the induction of tremor and



rigidity with tremorine, a cholinomimetic, first described by Everett (1956). These symptoms resemble, remarkably, those of the condition known as Parkinson's disease (Friedman and Everett, 1964). The symptoms of this hypermotor disease can be alleviated primarily through the use of cholinolytics which serve to depress central cholinergic function. Collier and Mitchell (1966-b) presented very convincing evidence, when they found that motor activity of a conscious animal was associated with a high rate of release of acetylcholine from the cortex and that inactivity was accompanied by a precipitous drop in acetylcholine output. Therefore, in contrast to the generally accepted linear relationship between cholinergic function and motor behavior, intergenerically, whole brain acetylcholine levels and motor performance are found to be inversely related.

Several speculative explanations do present themselves. The higher amount of acetylcholine in the less active mice genera may represent a greater number of inhibitory or regulatory cholinergic neurons than are found in Mus or Microtus. This would result in more inhibition of motor behavior in Peromyscus, Perognathus and Onychomys and less in Mus and Microtus. Alternatively, high acetylcholine may reflect a high degree of central cholinergic neuronal activity. Neuronal activity may have increased to such an extent and for such a long time that cholinceptive desensitization block may have resulted. This would, therefore, be exemplified by a decreased motor output. This latter possibility could be tested by determining relative cholinceptive sensitivities of the different mice genera. Evidence supporting either the presence of cholinergic inhibitory neurons or of depressed sensitivity of cholinceptive neurons is offered by the work of Scudder, et al (1966). They found that Perognathus

and Peromyscus are less sensitive to electroconvulsive shock, while Mus and Microtus are highly sensitive. This is of importance since the onset of seizure activity has been related to a release of acetylcholine (Naruse, et al, 1960; and Takahashi, et al, 1961).

e. Learning Performance

Karczmar and Scudder (1966 and 1967) reported that Mus mice were considered the best performers in avoidance learning, followed by Peromyscus and Onychomys mice, while Microtus did not improve in its performance at all. With the exception of Microtus, the best performers were found to have the least whole brain acetylcholine levels. However, again, this association of low levels of acetylcholine with good performers seems contrary to the general findings of other investigators. Anticholinergics are known to drastically interfere with the learning process (Longo, 1966), thereby implying a certain necessity for proper cholinergic functioning in the learning process. By the same token overstimulation of the cholinergic system, eg. by nicotine or an anticholinesterase can be equally detrimental to learning (Bovet, et al, 1966, 1967 and 1969; Bures, et al, 1962; Bures, 1968; Deutsch and Lutzky, 1967; and Pfeiffer and Jenney, 1957), while small doses of an anticholinesterase facilitate learning (Scudder, unpublished data). Therefore, one possible explanation for the present data is that the best performers, namely Mus, have a level of cholinergic activity best suited for learning; increasing this level of activity, as is Onychomys or Peromyscus, or decreasing cholinergic activity, as in Microtus, are both deviating from and detrimental to the optimum level of activity for learning. The basic assumption of this theory, that is,

that an optimum level of activation is required for maximum performance, is modeled after the "optimum level" theory of Malmö (1966). A possible way in which the degree of cholinergic activity could influence learning in these diverse genera of mice is by its regulation of exploratory, and possibly stereotypic and freezing, behaviors. Rosenzweig, et al (1960) equated intelligence with an ability to test out novel solutions to a problem. An animal exhibiting a high degree of exploratory behavior, such as Mus, would engage in many responses and would, consequently, be expected to "happen upon" the correct solution leading to an early solution. In contrast, low exploration would limit the chances of rapidly arriving at the proper response. This latter situation is exemplified by Onychomys, Peromyscus and Microtus mice, all of which exhibit relatively low exploration and poor learning. In this respect, the levels of acetylcholine in Mus optimize their exploratory behavior, thereby facilitating their learning performance. The lower acetylcholine levels of Microtus and the higher levels of Peromyscus and Onychomys do not optimize exploration in these animals. Consequently, their performances at solving the training situation is equally depressed. In addition to the factor of exploration, the tendency for Peromyscus and Onychomys to exhibit stereotypic behavior may result in these mice fixing upon a certain response, even though it is an inappropriate one, thereby hindering learning. Again, the cholinergic system could be involved here since high acetylcholine levels were associated with such stereotypy and freezing, as well as with low learning performance.

f. Electroconvulsive Seizure Latency

Reports in the literature have implicated high brain acetylcholine levels as a factor predisposing animals to seizure (Naruse, et al, 1960) and

as a regulator of seizure intensity and duration (Takahashi, et al, 1961). There is no apparent relationship between the level of acetylcholine and threshold of convulsion (indicated in part by latency to seizure) (Naruse, et al, 1960; and Takahashi, et al, 1961). However, the present genetic study has found whole brain acetylcholine levels to vary directly with the latency of electroshock seizure (Karczmar and Scudder, 1967; and Scudder, et al, 1966). The Mus and Microtus mice have the lowest acetylcholine levels and the shortest electroshock latencies; Peromyscus and Perognathus exhibit the longest latencies to electroshock seizure and also have the most brain acetylcholine. Since the biogenic amines and serotonin have been so strongly intimated to serve a regulating function of seizure latencies (Schlesinger, et al, 1965 and 1968; Scudder, et al, 1966; and Toman and Everett, 1958) this effect of regulating seizure latency in these different genera of mice is probably due to the NE and/or 5-HT, which parallel the whole brain levels of acetylcholine in these mice (vide supra), as Scudder, et al (1966) had suggested. The concimittant association of acetylcholine here might reflect the inherent balance of the cholinergic system and the adrenergic and/or serotonergic systems (Aprison, 1962; and Carlton, 1963). On the other hand, the cholinergic system may play a more vital role in controlling seizure latency in these genera of mice. One possibility is that the level of acetylcholine reflects the presence of a considerable number of cholinergic inhibitory circuits (by genetic design). The large number of inhibitory circuits, therefore, in Peromyscus and Perognathus mice would serve to offset the induction of seizure for a longer interval of time (electroshock latency) than in Mus or Microtus which have a proportionately lower number of inhibitory cholinergic circuits. In support of such a possibility,

Carlton (1963) had postulated an inhibitory function for central cholinergic circuits which would relate, possibly, to the longer latency period of high acetylcholine mice. Additional support can be found in the work of Wooley, et al (1963). They concluded that rats with high acetylcholine and cholinesterase undergo a seizure of less intensity compared to rats with less cholinergic components. This finding also supports an alternative possibility. The high acetylcholine may reflect overactive cholinergic neurons which have reached the point of depolarization block, i.e. inhibition of cholinceptive neurons due to overstimulation by acetylcholine. This would serve to decrease the effectiveness of electrically released acetylcholine, which is associated with the initiation of a seizure (Naruse, et al, 1960; and Takahashi, et al, 1961). Since such hyperactivity of the cholinergic neurons would not occur at the lower levels of acetylcholine, these corresponding mice genera, Mus and Microtus would be more sensitive to seizure, as in fact they are.

#### D. CONCLUSION

A preponderance of cholinergic circuits defines an animal that will be rather non-aggressive; timid and rather fearful. It would not tend to explore the environment readily. It would have a highly "emotional" and inflexible personality, exemplified by its stereotypic and freezing behaviors, and relatively poor learning performance. On the other hand, a highly exploratory and aggressive animal with a marked ability to adapt to the environment would exhibit comparatively few central cholinergic circuits. Obviously, however, the cholinergic system is not the only requirement for the expression of these behavioral traits. An interaction with other neurochemical systems would more closely define the basis of

differentiation of behavioral patterns, since each basic unit of brain, the neuron, contains a myriad of terminal boutons representing a complex of neurochemical interactions. Nevertheless, within this genetic sampling of mice, these were the typical behavioral responses associated with the particular concentrations of brain acetylcholine. It remains for further experimentation to define the extent of interaction of these biochemical systems.

## SECTION II

BEHAVIORAL AND DRUG INDUCED CHANGES OF ACETYLCHOLINE LEVELS IN  
ANATOMICALLY DISTINCT BRAIN REGIONS OF THE LABORATORY MOUSE

## CHAPTER VI

INTRODUCTION TO BEHAVIORAL AND DRUG INDUCED CHANGES OF ACETYLCHOLINE LEVELS  
IN ANATOMICALLY DISTINCT BRAIN REGIONS OF THE LABORATORY MOUSE

Genetic studies represent only one of many, more traditional, experimental attacks on the perplexing problem of the neurochemical basis of behavior (Rosenzweig, et al, 1960). The use of experimental manipulation and measurement of biochemical events and of behavioral traits and, subsequently, correlating these two parameters has allowed the formulation of several concepts concerning the cholinergic system. These methods of experimentation are used in this section of the thesis. However, several guidelines must be followed in evaluating data relating brain levels of acetylcholine and states of behavior, in order to avoid misinterpretation.

A necessary participation of the cholinergic system in maintenance of states of arousal has been deduced from the countless verifications of the original findings of Crossland and Merrick (1954), Elliott, et al (1950), Richter and Crossland (1949) and Tobias, et al (1946), that brain acetylcholine levels vary inversely with the degree of functional activity of the brain (Giarman and Pepeu, 1962; Takahashi and Aprison, 1964; and Toru, et al, 1966). Acute convulsive activity, induced either by drugs or electroshock, results in a transient but significant decrease in recoverable brain acetylcholine (Richter and Crossland, 1949; and Takahashi and Aprison, 1964). Milder states of drug induced behavioral excitation also result in a smaller decrease in brain acetylcholine (Aprison, et al, 1968; and Toru, et al, 1966). Sleep, tranquilization or depression increases acetylcholine concentration (Giarman and Pepeu, 1962; and Toru, et al, 1966). Maximal



increases in central acetylcholine are found in deep anesthesia (Crossland and Merrick, 1954; and Crossland and Slater, 1968). An explanation for such changes in acetylcholine was offered by Elliott, et al (1950). He proposed that influences depressing neuronal activity of the brain might be expected to decrease the release and subsequent destruction of acetylcholine, thereby allowing acetylcholine reserves to be built up. Conversely, increasing nervous activity might be expected to diminish acetylcholine stores. This original postulate received verification from MacIntosh and Oborin (1953) who demonstrated that during anesthesia the release of brain acetylcholine into a perfusion system was drastically reduced. Subsequently, Collier and Mitchell (1966-a and b and 1967), and Mitchell (1963), using the perfusion-cup method, described earlier (see General Introduction), correlated activity and convulsions with a high rate of release of acetylcholine and inactivity with a drop in acetylcholine output. Sleep was accompanied by an immediate fall in acetylcholine release.

This explanation of the acetylcholine changes in different states of neuronal activity is intuitively correct. However, in certain instances some revision of this theory is required. During behavioral excitation, environmental stimuli impose an increased demand on the cholinergic system to release acetylcholine. Acute stimulation can be satisfied by the ready available supply and normal store of neuronal acetylcholine. However, if such a stimulus is constant and chronic, the system might adapt or respond by increasing the normal rate of synthesis or by increasing the number of synaptic sites releasing the cholinergic transmitter, both responses resulting in a positive increase in recoverable brain acetylcholine. Therefore, it is theoretically possible to argue that chronic behavioral

excitation might be correlated with an increased amount of acetylcholine. On the other hand, prolonged states of depression, or lack of stimuli, could be adapted to by decreasing either the rate of acetylcholine synthesis and/or the number of functioning cholinergic synapses. This would experimentally be described as a decrease in recoverable acetylcholine. Evidence indicating the possibility of such events can be found in the work of Rosenzweig, et al (1968). Exposing animals to an environment in which they are constantly receiving environmental stimulation results in an increase in brain level of cholinesterase activity. In these instances cholinesterase is indicative of the level of acetylcholine, since it was found that changing acetylcholine levels induces a parallel change in cholinesterase (Burkhalter, et al, 1957). Animals deprived of such constant stimulation had both lower cholinesterase activity and lower acetylcholine levels. Consequently, in discussing relationships between states of behavioral activity and brain acetylcholine levels, the length of time the animal was subjected to the particular degree of behavioral activity must be considered.

In the face of what is known as the "inverted-U theory", the "activation theory" or the "optimum level theory", the inverse correlation of acetylcholine levels with states of functional activity (remembering the conditions imposed by acute or prolonged states of activity) is applicable only under careful scrutiny. The major postulate of the optimum level theory is that "there is an optimal condition or point of activation for best performance; on either side of this point, performance is relatively impaired" (Malmo, 1966). This optimum level may fluctuate at times. As applied to the cholinergic system and behavior, this theory holds that there is a level of activity of the cholinergic system at which behavioral performance is

optimized. Either an increase or decrease from this optimum level of cholinergic activity will depress behavioral performance. This application of the optimum level theory receives support from Bohdaneky and Jarvik (1967) who found that learning ability is depressed by both physostigmine and atropine. The genetic studies of Bovet, et al, (1966 and 1969) in which nicotine facilitated poor learners but depressed good learners gives further support to the idea of an optimum cholinergic activity for maximum performance. Also, Bures (1968) reports that excessive stimulation of the cholinergic system results in depressed motor output and some degree of sedation, while anticholinergics have opposite effects. Such hyperactivity of the cholinergic system could possibly disrupt behavioral performance initially by altering the optimum level of coordination of synaptic events (Roberts, 1966). At this stage of behavioral disruption neural activity is increased while effective performance is impaired. This was experimentally shown by Russell (1958 ). Further increases in cholinergic activation could then depress neuronal firing by inhibiting the cholinergic receptor via a depolarization block (Deutsch and Lutzky, 1967; Reeves, 1966; Roberts, 1966 and Wilson and Altamirano, 1956). At this point performance is abolished and neural activity is beginning to decline. However, this rather severe state of general cholinergic activity is usually brought about by a potent anticholinesterase ( Deutsch and Lutzky, 1967 ). Consequently, behavioral depression could represent either a state of increased or decreased neuronal activity resulting from either an increased or decreased cholinergic activity. To obtain an idea of the functional meaning of an increase or decrease in brain acetylcholine, as related to a certain observable behavior, one must draw upon all information available to ascertain the state

of neuronal activity.

in addition, it should not be assumed that the cholinergic system alone functions to regulate behavior. A number of theories have been proposed as to the interaction of several central biochemical systems in regulating animal behavior (Aprison, 1962; Aprison and Takahashi, 1965; Brodie, et al, 1959; Carlton, 1963 and Hess, 1954). However, no definite mode of interaction has been defined. The wealth of evidence does point to the existence of such relationships between the cholinergic system and other biochemical systems (Toru, et al, 1966).

Using these guidelines, the present section of this thesis will investigate the cholinergic system in association with certain states of behavior, eg., learning, stress, isolation, aggression and convulsion, and attempt to describe it's functional relationship to each of these. Subtle changes in levels of the cholinergic transmitter consequent to certain states of behavior may occur in discrete brain areas, only. Since small, but significant regional changes in acetylcholine concentration could be hidden in determinations of whole brain levels (Aprison, 1962; Bowers, et al, 1966; Singh and Malhotra, 1964 and Toru, et al, 1966), regional brain levels of acetylcholine are presented here.

CHAPTER VII

EFFECTS OF LEARNING AND SHOCK-STRESS ON REGIONAL BRAIN

ACETYLCHOLINE LEVELS OF MUS MUSCULUS CFI

## A. INTRODUCTION

### a. A Physiological Theory Of Learning

Learning is a number of processes concerned with a response to environmental stimuli. To respond with a behavior is one of the most important ways an animal reacts to it's environment (Fuller, 1964; and Scott and Fredericson, 1951). Behavioral changes are generally adaptive or homeostatic to the environmental changes in that they allow for the maximal functional efficiency attainable by the organism under the circumstances (Roberts, 1966). As such a "behavior" can be defined as any response, of which the organism is capable, that will effectively cope with the environmental challenge. The mechanical or systemic factors determining the most effective way of dealing with the situation, or the point of maximal functional efficiency, are at present poorly understood. Speculatively, such a decision-making system could reside in the coordinated attempts of subsystems to maintain steady states throughout the organism; or, it may comprise the interaction of controlling influences from "reward" or "punishment" centers (Olds, 1962). The organism, in any case, is usually characterized by an ability to respond to a stimulus with a display of the most appropriate behavior of its physical repertoire which actively removes or modifies a noxious stimulus or furthers conditions which will increase a pleasant stimulus in the most efficient manner (Roberts, 1966). In developing or evolving such an appropriate behavior, the organism continually perfects and tests various patterns of behavior within it's genetically and developmentally endowed limits. These limits, on occasion, may allow for the synthesis of an entirely new behavior. The sequence in which the organism will test behavioral sets in response to a novel or challenging stimulus

will depend upon which behaviors have been previously most frequently used. According to current nerve-circuit theories, an increase in solidarity of neuronal connectivity, as expressed by increased probabilities of specific synaptic transmissions, occurs with use (Roberts, 1966). Therefore, the most used behavioral set will be most readily activated, followed by the next most used behavior, etc. As each behavioral set is tried, if unsuccessful, this particular behavior may be depressed by an elaborate inhibitory feedback system (Roberts, 1966) and the next behaviors, or combination of behaviors, may be tried. Through such trial and error, the animal should ultimately arrive at that pattern of behavior which is most effective in maintaining maximum efficiency of the organism. It should be realized that even though the organism adopts this behavior as the most satisfactory, it may not actually be the best response in reference to some parameters of importance to it. Some evidence is available which indicates this suppression of inappropriate responses to be a function of the reticular formation (Rosvold and Mishkin, 1961), under the tonic directive influence of the cortex (Adey, et al, 1957; and Bures, et al, 1963). The reticular formation has been shown capable of inhibitory and facilitatory functions (Magoun, 1963). Bures, et al (1963) have demonstrated the cortex to have a tonic effect on these reticular functions. Consequently, the decreased performance ability in monkeys after frontal lesions, described by Miles (1964) and Konorski (1961) as being due to a lack of ability to inhibit old, inappropriate responses was interpreted by Rosvola and Mishkin (1961) as being due to a failure of the reticular system to suppress the inappropriate responses. Changing the neuronal network to favor a certain pathway enhances the likelihood that the next stimulus will activate the involved neurons as a whole, unit circuit. Having thus

established the appropriate neuronal connections which elicit the most efficient behavior, subsequent reception of the same stimulus will tend to generate this learned behavioral response in deference to other inappropriate responses. As consolidation of the appropriate neuronal patterns is attained, inhibition of the unrewarded or inappropriate behaviors, needed during the trial and error period, is no longer necessary. Consequently, as the learned behavior becomes more well-learned, the original inhibitory system becomes less active until a minimum of inhibition of the unrewarded behaviors is necessary. Such an increase in neuronal connectivity with use probably lies at the basis of development of memory and all types of learning (Roberts, 1966).

Since the neuronal connections\* involve synapses, and since synaptic function is related to the release of a chemical transmitter, increasing neuronal connectivity may be attained by the liberation of more transmitter per impulse at the appropriate synaptic connections (Kaplan, 1962). This would then produce a greater effect upon the post-synaptic membrane. Two methods to increase the amount of functional transmitter might be to increase synthesis or release of the transmitter within the same nerve terminal. While this would be the basis for momentary or brief behavioral change of a rapidly effective nature, a more permanent change might involve growth of neuronal elements to increase the amount of physical contiguity between the pre- and postsynaptic sites. This might necessitate more glial material to furnish the metabolic needs (Roberts, 1966). Such a mechanism has received some support from the finding by Altman and Das (1964) of a larger number of new glial cells in animals in a highly stimulating environment, compared to the amount of fresh glial cells in animals from a relatively impoverished



environment.

The problem of how neuronal connectivity is enhanced at only specific appropriate synaptic units is yet to be decided. The fact that general excitation of the brain, eg. by electroconvulsive seizure (Thomson, et al, 1961), as well as general depression (Garg and Holland, 1967), will depress the learning process implies that maintained activation of the proper synapses may be necessary to restrict the action of increasing the connectivity to these select synapses (Garg and Holland, 1967; McGaugh, 1966; and Pearlman, et al, 1961). It is possible that induced neuronal connectivity may be promoted by some circulating nerve growth factor.

The available data indicates that activation of the hypothalamic-hypophyseal system facilitates the making of the maximum possible connectivities in active neural circuits (Roberts, 1966). This becomes of special importance when one considers the fact that ACTH has long been known to delay the extinction of learned behaviors (Bohus and DeWied, 1966), and that thyroid hormone and growth hormone have been shown to influence neural growth (Eayers, 1961).

#### b. The Learning Process And The Central Cholinergic System

The vital involvement of the cholinergic system in the learning process is supported by many findings. The administration of cholinolytic drugs, such as atropine, scopolamine or benactyzine, which antagonize the action of acetylcholine (Ach), to animals in various kinds of learning situations has long been known to impair performance (Longo, 1966; Meyers and Domino, 1964; and Pazzagli and Pepeu, 1964). This amnestic effect was observed only in animals trained for a short period but the drugs were ineffective (except at very high doses) in well-trained animals (Buresova,

et al, 1964; Herz, 1960; and Pazzagli and Pepeu, 1964). Anticholinergics administered during the period of training are also found to disrupt acquisition of a learned behavior (Bohdanecky and Jarvik, 1967; Herz, 1960; Meyers, et al, 1964; and Meyers, 1965). However, some reports have shown cholinolytics to enhance acquisition of a learned behavior (Bignami, 1964).

Cholinergic stimulants, such as nicotine (Bovet, et al, 1966 and 1969) and physostigmine (Bohdanecky and Jarvik, 1967), have been reported to have variable effects (both facilitation and impairment) on acquisition (Bures, et al, 1962). Similarly, nicotine, physostigmine and DFP may facilitate (Deutsch and Lutzky, 1967; Morrison, 1967; and Russell, 1958) or depress (Bures, et al, 1962; Bures, 1968; Deutsch and Lutzky, 1967; Morrison, 1967; and Pfeiffer and Jenney, 1957) retention performance of a previously learned behavior. Such variable effects may reflect either an optimum level of cholinergic functioning or the extent of consolidation attained at the time of drug administration. At any rate, the fact that the process of learning (acquisition and consolidation) is consistently affected by cholinergic drugs strongly indicates that the central cholinergic system plays a vital role in regard to the appropriate response to environmental stimuli. Furthermore, the variable effects of facilitation or impairment of learning induced by the administration of cholinergic drugs does provide support for the hypothesis that "an optimum level of cholinergic functioning is necessary for efficient learning" (Bohdanecky and Jarvik, 1967).

On the basis of central cholinesterase activity and acetylcholine levels, Rosenzweig, et al (1962) have also concluded that the cholinergic system participates in learning and the ability to adapt to the environment. Rats reared in an enriched environment contained a more active central

cholinergic system than rats raised in an impoverished situation. The animals with more cholinesterase and acetylcholine, which were raised in an enriched environment, were found to surpass the second, impoverished group of animals in learning ability. Sklyarov and Kononenko (1963) also determining cholinesterase activity concluded that cholinergic activity is associated with conditioning behavior. Attempts to relate changes in brain levels of acetylcholine with learning behavior are still very scarce and have met with limited success. Toru, et al (1966), using a shock-avoidance conditioning apparatus, found no difference in the levels of acetylcholine in any of three brain parts ( telencephalon; midbrain-diencephalon; pons-medulla ) between naive rats, rats with shock-avoidance training or rats exposed to shock but without avoidance training. However, these investigators did associate decreasing cholinergic functioning with depression and decreased avoidance behavior, while greater cholinergic activity was related to behavioral excitation and increased avoidance behavior. Pazzagli and Pepeu (1964) were able to find that scopolamine caused a decrease in brain acetylcholine which was directly proportional to the intensity of the amnesic effect. It should be mentioned here that the decrease in brain acetylcholine following the acute administration of an anticholinergic (scopolamine) is not indicative of an activation or arousal. While effectively blocking any stimulation of the receptor, scopolamine increases release and subsequent destruction of the presynaptic acetylcholine. It is of some interest that the effect on brain acetylcholine and the amnesic effect tended to disappear with repeated doses of scopolamine. However, Slater (1968) questions this relation of acetylcholine levels and amnesia, since drugs, which decrease acetylcholine levels but which are not cholinolytic (as are atropine and

scopolamine) do not affect learning proportionately. He attributes the amnesic properties of atropine and scopolamine solely to their blocking ability at cholinergic receptors, not to their effect on acetylcholine levels. However, it is also possible that the drugs used by Slater, triethylcholine and hemicholinium-3, may not depress cholinergic function sufficiently at the particular synapses involved in learning which may be affected by atropine and scopolamine.

More indirect evidence linking learning with central cholinergic function is found in the fact that the ascending reticular formation, which was suggested to participate in the learning process, is thought to function via a cholinergic mechanism (Rinaldi and Himwich, 1955). Supporting this proposition is the finding by Collier and Mitchell (1966-a and b, and 1967) that stimulation of the diffuse and specific reticular formation result in an increased release of acetylcholine from the cortex.

One of the active components of the pituitary in facilitating the learning process has been shown to be ACTH (Bohus and DeWied, 1966). In hypophysectomized animals, the impairment in performance of a recently learned behavior can be reinstated upon the administration of ACTH (Bohus and DeWied, 1966; and Weiss, et al, 1969). Hedge and Smelik (1968) have demonstrated the release of ACTH in the intact animal to be intimately related to a central cholinergic mechanism. Furthermore, hypophysectomy greatly limits the degree of central synthesis of acetylcholine (Torda and Wolff, 1950). However, replacement of ACTH, but not several other pituitary hormones, restored the ability of brain tissue to synthesize normal amounts of acetylcholine (Torda and Wolff, 1952-a). Since ACTH was found unable to increase the activity of choline acetylase in vitro, it was concluded that

ACTH facilitates acetylcholine synthesis in vivo by inducing the availability of proper substrate (Torda and Wolff, 1952-b). Therefore, ACTH may facilitate the learning process possibly by enhancing central cholinergic activity at strategic locations.

Although most available evidence indicates that the cholinergic system is involved in the process of learning, the mechanism whereby acetylcholine functions in learning is obscure.

One theory that does satisfy some of the available data was proposed by Carlton (1963). He postulated the cholinergic system to be responsible for selectively inhibiting the non-rewarded (or maladaptive) responses during the learning process. (According to this theory the cholinergic system itself is not inhibitory but stimulates an inhibitory system). Such a function could be postulated for the reticular activating system which Collier and Mitchell (1966-a and b, and 1967) have shown to be cholinergic and which has been suggested to be capable of inhibiting behavioral responses (Rosvold and Mishkin, 1961).

However, other possibilities exist.. Deutsch and Lutzky (1967) have proposed that cholinergic neurons are instrumental in establishing or activating the learned behavior itself. As the behavior becomes more well-learned, the proper cholinergic synapses will grow or increase proportionately. As a result, the learned behavior would be more susceptible to disruption by anticholinergic drugs early in the learning process, when cholinergic connections are weak, and less susceptible late in the learning process or in well-trained animals. That this modification of sensitivity occurs is clearly substantiated by the literature (Buresova, et al, 1964; Herz, 1960; and Pazzagli and Pepeu, 1964). Of course, such sensitivity

changes could also support Carlton's (1963) proposal that cholinergic activity serves to depress inappropriate responses until the correct response is neuronally consolidated.

c. Habituation

In learning to respond properly to the environment, an animal will initially respond reflexly to any novel stimulus by orienting its attention to it (Zinny and Kienstra, 1967). Exploration of a novel environment is considered a vital part of the orienting reflex (Carlton, 1968). Subsequent presentation of the same stimulus will either engender continued orientation and additional reactions (leading to a learning of the appropriate response) or a decrease in intensity of the orienting reflex, including a depression of exploration. This latter phenomenon is called habituation and is thought to result from inhibition of the reception of the sensory stimulus via negative feedbacks. Roberts (1966) and Sokolov (1960) consider such negative feedback to originate primarily from cortical and also thalamic reticular sites, and to restrict the sensory input at various locations including the receptor site, the central sensory nuclei, and other central relay stations. The effectiveness of these negative feedback mechanisms in reducing (i.e. habituating the organism to) sensory input is a function of stimulus intensity as well as degree of "significance" to the organism (op cit; Carlton, 1963). Significant stimuli include food and water, or any perception that the animal associates with reward or pain. These feedback mechanisms for habituation go into play immediately upon reception of any sensory input. If the feedback system is effective in preventing sensory input from reaching the higher brain centres, habituation is said to have occurred. In this sense then habituation is another form of adaptation or learning. Carlton (1963) has implicated the

inhibitory action of the cholinergic system as being essential for habituation to occur and has related habituation to learning. This is in keeping with Robert's (1966) contention that habituation does come under the realm of learning and is a function of inhibitory systems. That habituation is caused by a mechanism similar or identical to that causing learned behavior is further suggested by the fact that sound and air-puff habituations are transferable from animal to animal in the form of some thus far unknown brain component as are other types of learned behavior, such as conditioned behaviors (Ungar, 1967).

#### d. Chronic Stress

Habituation is usually successful in response to mild, innocuous stimuli but not to strong and/or "significant" stimuli. To the latter, the animal reacts with a display of a series of behaviors until the proper response is acquired. Until the animal arrives at the proper response, which changes the environment appropriately, the stimulus or environmental challenge is constantly impinging upon the central nervous system of this animal. A classical method of conditioning animals is to use mild electric shock as a negative reinforcement. During the training sessions, the animal is exposed to the stress of shock and, eventually, the animal will exhibit an ensuing series of behaviors in an attempt to reduce this stress (superimposed on the initially unsuccessful attempts to physiologically habituate to this stress).

Until the stimulus is lessened, it is stressful to the organism in several ways. Firstly, it threatens the homeostasis of the organism and obliges it to respond with secretory and motor patterns. Secondly, since initially activated habituating negative feedback systems were ineffective in restricting its input, this stimulus is constantly impinging upon the

central nervous system activating a host of neuronal pathways including those of the hypothalamo-hypophyseal system (Roberts, 1966). In the usual method of conditioning, animals are trained to show particular behavior on cue to avoid the negative conditioning stimulus, a mild electric shock. These animals are apparently being stressed and show a typical alarm reaction, eg., squeaking, wild running and jumping, urination and defecation (Mallov and witt, 1961 ). However, if such animals modify their behavior and thus successfully minimize the shock, stress is reduced. But the question arises, if the animal is not allowed to behaviorally modify the shock, how long will the animal continue to test behavioral solutions? Is the physiological make-up of the organism such that behavioral defensive maneuvers cease after a time ? The exposure to prolonged or chronic stress has been shown to induce several characteristic physiological, anatomical and biochemical changes.

#### 1. EEG and stress

Few attempts have been made to determine whether an animal, exposed to a solvable conditioning procedure exhibits more, less, or no reactions to the stress of the conditioning situation, as compared to an animal exposed to a similar, but unsolvable, conditioning procedure. One such study compared the EEG patterns of a group allowed to learn and of a frustrated group prevented from learning (Freedman, et al, 1966). There were found clearly distinct differences in states of arousal of the two groups. As learning progressed, states of arousal, based on EEG patterns, decreased, while the frustrated group demonstrated a continually increasing pattern of arousal. EEG patterns of the type demonstrated by the frustrated group have previously been related to heightened emotionality, stress or frustration



(Mundy-Castle, 1951 ).

ii. Biochemical correlates of stress

(1) The adrenergic and serotonergic systems, ACTH and glutathione

Biochemical evidence has also been reported which supports the above contention that an increased state of central excitation is maintained in the stressed animals. Stressful situations of various forms, including mild shock, have been correlated with a decrease in central norepinephrine levels (Barchas and Freedman, 1963; Corrodi, et al, 1968 and Ruther, et al, 1966) which is indicative of increased brain activation (Scudder, et al, 1966). A number of reports indirectly imply such a decrease in central norepinephrine. Stress induced activation of central sympathetic mechanisms can be detected by an increase in plasma free fatty acids (FFA) and an increase in urinary catecholamines, both due to an enhanced adrenal activation. An increase in urinary catecholamine excretion was found to result from intermittent electrical stimulation (Michalova, et al, 1966). Similarly, plasma FFA increase significantly during stressful situations (Khan, et al, 1964 a and b; Mallov and Witt, 1961 and Michalova, et al, 1966). Closely associated with the apparent activation of both the peripheral and central adrenergic systems in stress is the fact that shock increases the toxicity of amphetamine (Weiss, et al, 1961). The authors suggested this to be due possibly to the associated increased activation of the adrenergic system.

Another characteristic feature associated with stress is the wellknown increase in ACTH release from the pituitary (Moore, 1966). The central control of ACTH was found to depend upon activation of a cholinergic component (Hedge and Smelik, 1968). Furthermore, ACTH release occurs without exception with every stressing event, even when such events are

repetitive (Sachar, et al, 1967).

Generally, the data on the state of the serotonergic system during stress support the idea that its activity is increased, similar to that of the adrenergic system. Liberson, et al (1964) and Corrodi, et al (1968) showed that central levels of 5-HT are decreased in animals exposed to stress. Liberson, et al (1964) found this decrease to be more prominent in the cortex and hippocampus than in the brain stem and cerebellum. An increase in brain 5-HT was, however, found by Ruther, et al (1966) and Barchas and Freedman (1963). The stress induced changes in serotonin were interpreted by Welch and Welch (1968) and Eleftheriou and Church (1968) as being indicative of an increase, not a decrease, in serotonergic activity. Welch and Welch based this decision on the finding of increased amounts of 5-hydroxy-indoleacetic acid, a metabolite of 5-HT. Eleftheriou and Church, measuring the activity of 5-hydroxy-tryptophan decarboxylase, the rate-limiting enzyme in the synthesis of 5-HT, found the frontal cortex to exhibit a 300% increase in its activity.

A further biochemical distinction of stressed animals is that they have decreased blood and brain levels of glutathione, which partly functions normally to maintain available supplies of sulfhydryl compounds, such as Coenzyme A (Varma, et al, 1968). The authors attributed this decrease to an increased activity of the glutathione system.

Stressed animals have also recently been found to excrete a urinary substance, the odor of which is distinguishable by other rats from the urine of non-stressed rats (Valenta and Rigby, 1968).

## (2) The cholinergic system during stressful situations

Toru, et al (1966) found no change in acetylcholine levels of

three brain parts among rats that received shock but were not allowed to avoid, rats that received shock and were allowed to avoid, and naive controls. This seems contrary to the report by Krause, et al, (1964) that both cholinergic and adrenergic activity increased in a telencephalic-diencephalic region of the mouse brain during emotional excitation (conditioned fear). These authors conditioned mice to respond to a light in the same manner as they responded to an electric shock. There was a decrease of both acetylcholine and norepinephrine in these conditioned mice, which the authors interpreted as indicative of increased activity. The only additional study relating acetylcholine and stress was that of Bowers, et al, (1966); they found no significant changes of acetylcholine in any of three brain regions in stressed animals.

### iii. Stress-associated behaviors

The physiological and biochemical evidence at present indicates that the stressed animal is in a state of increased central activation. However, in comparison, the behavioral state of such animals seems somewhat contradictory. Animals subjected to inescapable applications of repeated stress, such as mild electric shock, respond initially by squeaking, jumping, wild running, defecating and urinating (Mallov and Witt, 1961). Such reaction to the shock accompanies various other defensive patterns of behavior, such as escape and fighting (Scott and Fredericson, 1951). This heightened state of central stimulation is carried over to the inter-shock interval in the form of exploratory behavior. However, upon further application of repeated stress, the animal adopts a very characteristic set of behaviors. The reaction to repetitive shock is still qualitatively similar to the original type responses but quantitatively the shock appears to cause less discomfort (Mallov and

Witt, 1961). Brown-Grant, et al (1954) made similar observations using the rabbit. Immediately following each shock the animal exhibits a state of tonic immobility, a type of freezing or catatonic behavior, for a variable length of time. This is followed by an apparent, behavioral depression or withdrawal during the remainder of the intershock interval during which the animal lies quietly but not asleep (Khan, et al, 1964-a and b; Mallov and Witt, 1961; and Ruther, et al, 1966). Oken, et al (1960) and Grinker (1966) consider such behavioral withdrawal and apparent depression to be an alternative defense response to continued stress and anxiety, when other behavioral adaptations are ineffective. That this final defense is at least partially effective is indicated by the fact that a decreased responsiveness to stimuli results from successive administrations of shock or other stresses (Grinker, 1966; Holmberg, 1954; and Lutz, 1967).

#### iv. Physiological theories of stress

As implied by the physiological and biochemical data reported earlier, this behavioral loss of responsiveness is not necessarily a correlate of central nervous system depression, and may in fact, occur during hyperexcitability (Winters, 1967). In addition, the catatonic behavior occurring during the later adapted period of stress, is a typical prodromal symptom of convulsive seizures (Naruse, et al, 1960). "Freezing" behavior is also considered indicative of a high degree of emotional arousal (Bovet, et al, 1969). Furthermore, on the basis of high adrenal cortical hormonal blood levels, Board, et al, (1956) and Board, et al, (1957) have shown that an individual, under previous chronic stress and appearing withdrawn and depressed, is really biologically active. Therefore, the physiological make-up of an animal, incapable of behaviorally adapting to stress, restricts

the central reception of the stressing stimulus (Oken, et al, 1960).

According to Roberts (1966), this restriction could be similar to habituation. While habituation is usually observed in connection with mild, non-significant stimuli, it should not be assumed that the negative feedback system becomes inactive if it does not initially succeed in restricting sensory input. Rather, this process seems to be automatically activated upon reception of sensory stimuli and continues, unless it is actively inhibited from higher centers, throughout the period of sensory reception. The negative feedback system associated with sensory-input increases with use of the sensory pathways. A strong sensory input (shock) may have activated aspects of the negative feedback system which, gradually, effectively blocked the stimulus input. Winters (1967) has supported this idea and proposed a model indicating that sensory-input systems have an increased modulation during excited states.

An alternative suggestion could be that increased neural activity, induced by the continual stress, eventually surpassed the optimal level of activation associated with maximum performance of most behaviors (Malmo, 1966) and opposing systems may effectively cancel one another out. A third possibility to explain the dichotomy between increased neuronal activity but decreased behavioral expression is based on the proposed learning process itself. Since initial habituation was unsuccessful in preventing the entrance of the noxious stimulus, early exposure to this stress stimulus induced the normal learning process. As each behavior failed to reduce the adverse stimulus, it was eventually inhibited by a negative feedback (the reticular system). Continual operation of this sequence of trial, error and inhibition of various behaviors culminated in inhibition or reduction of all behavioral acts resulting in a behaviorally withdrawn and behaviorally

depressed, but a centrally physiologically active, animal. The stress stimulus causes sufficient excitation of the brain to both release ACTH (Sacher, et al, 1967) and elicit a reduced alarm reaction to the shock itself (Mallov and Witt, 1961). This supports the latter possibility that active feedback inhibition of behavior occurs. However, the fact that stress-inhibited thyroid activity returns to control levels before cessation of stress and that this occurs in association with a reduction in severity of response to the stress indicates that some restriction of input to the brain is also occurring (Brown-Grant, et al, 1954). Regardless of the exact physiological mechanism, most theories regarding the response of an animal to stress do agree in the sense that there is some type of internal inhibition (Eriksen, 1966).

Even though the response adopted by the chronically stressed animal is partially effective in reducing the central reception of the shock, this withdrawal is a neurotic behavior, that is, maladaptive in the sense that it does not completely promote the well-being of the organism (Wolpe, 1966). The animal is withdrawn from the surroundings, apparently behaviorally depressed, with a diminished degree of responsiveness, and it may display "freezing" or catatonic behavior. Both "freezing" (Bovet, et al, 1969) and catatonia (Perez-Cruet, 1967) are frequently associated with the development of experimental neurosis.

#### v. Implications of the cholinergic system in stress

There are tenuous indications that a maladaptive response to chronic stress reflects the presence of a hyperactive cholinergic component. Although scanty biochemical evidence cited previously is inadequate to support the presence or absence of such a system in the response to prolonged stress,

states of tonic immobility, "freezing" behavior or mild catatonia in different mice genera has been associated with high levels of brain acetylcholine (confer section I). Ruther, et al, (1966) attributed the cataleptic behavior in stressed animals to some system other than adrenergic or serotonergic. Catalepsy is a frequent component of the prodromal symptoms of seizure (Naruse, et al, 1960) which has long been known to involve a significant release of acetylcholine (Crossland and Merrick, 1954; Elliott, et al, 1950; Richter and Crossland, 1949 and Tobias, et al, 1946). A cataleptic state can be induced in mice by administration of a number of different central cholinergic stimulants (Zetler, 1968). Successful antagonism of drug induced catalepsy (Zetler, 1968), as well as of catalepsy induced by brain lesions (Delini-Stula and Morpurgo, 1968), was accomplished with a number of anticholinergics, including imipramine which has been shown to have central anticholinergic effects (Toman, 1963). Furthermore, Ruther, et al, (1966) were able to show that in the presence of imipramine the catalepsy in stressed rats was reduced and hypermotor activity replaced the behavioral withdrawal and depression.

The research in this chapter will attempt to expand the current knowledge of the state of cholinergic activity associated with the stress of learning and during prolonged stress. This research will involve determinations of the levels of acetylcholine in three brain regions, the telencephalon, midbrain-diencephalon and pons-medulla (Aprison, et al, 1968; Toru, et al, 1966) of mice immediately after learning or stress.

## B. MATERIALS AND METHODS

### a. Animals

Determinations of acetylcholine levels were made on the brain of adult, male Mus musculus CF1 mice. These animals were purchased from Carworth farms, New Jersey, at an age of 55-60 days. They were housed in groups of 10 per cage and maintained in constant temperature and humidity chambers. Food and water were supplied ad libitum. At the time of use, body weights were approximately 30-40 grams.

Mice used in the hexobarbital study and in the scopolamine pilot study were adult, male Mus musculus SC1 obtained from Scientific Small Animals Farm, Illinois. The SC1 strain is a direct descendant of the CF1 strain. Reference to section 1 of this thesis shows these 2 strains to be very similar in regional levels of brain acetylcholine.

### b. Experimental Design

#### i. Training and shock-stress

Acetylcholine concentrations were determined in three groups of M.m.CF1 mice (each determination required the pooling of 5 mouse brain parts):

1) Trained Group - This group (total of 25 mice) was subjected to a solvable conditional-avoidance learning procedure using the climbing screen apparatus. The mice were sacrificed immediately after training.

2) Shock-Stressed Group - These mice (total of 20) were exposed to an unavoidable, shock-stress in the same climbing screen as used above, in other words, they were not allowed to avoid or escape the punishing electric shock. The mice were sacrificed immediately after the final shock.

3) Naive-Control Group- These mice (total of 25) received no treatment at all but were taken for sacrifice directly from their home cage.



Active avoidance conditioning of the animals was carried out using an automated climbing screen apparatus designed and built by Scudder (Scudder, et al, 1965) The apparatus consists of 6 small chambers, each with an entrance and exit door and connected by 5 inclined runways. The mouse is placed in the first base conditioning chamber. The automated training begins when the exit door of this chamber opens up to the first inclined grid. At the same time the entrance door of the second chamber opens. This series of events begins the 5 second warning period after which a mild shock is delivered to the grid of the base chamber. In this manner the animal is forced to escape from the shock by leaving the chamber and to enter the first inclined grid. The electric shock follows the mouse up the grid forcing it to enter the second chamber at which point the activation of photocells terminates the shock and closes the door behind the mouse now in the second chamber. After a rest period of 1 minute the same sequence of events is repeated consecutively as the mouse enters the third, fourth, fifth and sixth chambers. Upon entering the last chamber, the mouse is removed and replaced into the first chamber and the entire process is repeated again. One run through the climbing screen is considered to be one trial. Each animal is so processed 10 times. Therefore, a training session consists of 10 trials at least 5 tests per trial for a total of 50 individual tests per mouse per session. The time spent in each chamber, as well as the time interval from leaving one chamber until entering the next, is recorded automatically in tenths of a second.

The efficiency of this apparatus in training CFl mice to avoid the electric shock has been previously established (Scudder and Karczmar, 1967). Such learned avoidance behavior was confirmed specifically for the

mice used in the present experiment. However, while the mice did eventually learn to avoid, each animal was still exposed to a considerable amount of electric shock throughout the training session. The same climbing screen apparatus was used to shock the stressed group of mice, in order to treat the two groups of mice as similarly as possible. Each of the stressed mice was placed in the first conditioning chamber but the exit door was not allowed to open at any time during the period of stressing. The cycle of shocking was the same as that of training, previously described. A mild shock (average duration of 2 seconds) was delivered once every minute for 50 shocks. Since this stressed group of mice was not allowed to avoid, each mouse received the total 50 shocks. Therefore, in this respect it differed somewhat from the trained group which learned to avoid the shock, receiving on an average 10-15 shocks less than the stressed group.

Immediately after the last training test or shock episode, the animal was sacrificed by decapitation, the brain removed and sectioned into 4 parts, the telencephalon (T), the midbrain-diencephalon (M-D), cerebellum (C) and pons-medulla (P-M). Each brain section was rapidly frozen in liquid N<sub>2</sub>. A detailed description of excision of the brain and segmental division is found in chapter III of this thesis. Since pooling of brain tissue was necessary, the brain parts were kept frozen in liquid N<sub>2</sub> until all of the brains (5) were obtained. Due to the fact that each learning session required approximately one hour to complete, it was necessary therefore to collect brain tissue over a period of about 5 hours, one brain per hour. In order to account for any circadian effects on brain acetylcholine, the naive controls, as well as the stressed group, had to be sacrificed at times of the day closely approximating the times of day at which the trained animals

were sacrificed. To account for other possible interfering variables, such as age of the mice, the order in which determinations of acetylcholine were made in the three experimental groups was varied, eg., control, stressed, trained, stressed, control, trained, trained, etc.

The differences in mean acetylcholine concentration of each brain part between control and trained groups and between control and stressed groups were analyzed statistically for significance. The method of unpaired Student-t was used (Batson, 1956).

Acetylcholine concentrations were expressed in terms of acetylcholine HCl as micrograms/gram brain tissue.

#### ii. Effects of scopolamine on stress-induced behavior

To test the involvement of cholinergic activity in contributing to the stress-induced behaviors, an anticholinergic was given to pre-stressed mice. In this pilot study two groups of the white laboratory mouse were used:

- 1) Shock-stressed group (4) receiving scopolamine, 2mg/kg i.p.
- 2) Shock-stressed group (6) receiving physiological saline, i.p.

All the mice were individually stressed by repetitive shocks delivered in an isolation, shock-box, (Lehigh Valley Electronics). Fifty mild shocks (2 seconds duration) were given, one every minute. Behavioral observations included noting the presence or absence of jumping, squeaking and wild running in response to the shock. Also, between shocks mice were watched for "freezing" or catatonic posturing, exploratory activity or quiet resting. These latter observations were recorded on a present or absence basis.

After the initial fifty shock, 4 of the mice received 2mg/kg

scopolamine HBr, i.p., and 6 were injected with physiological saline, i.p. After 15 minutes (at which time scopolamine has an antiaggressive effect (Scudder, unpublished data), alters EEG tracings (Pupillo, unpublished data) and drastically alters brain acetylcholine levels (see chapter 9 of this thesis)), both groups of animals were exposed to an additional fifty inescapable shocks and the same behavioral observations made.

### iii. Hexobarbital sleeping time

Hexobarbital sleeping times were determined for 4 groups of M.m.SCl mice:

- 1) Trained Group - These mice (total of 10) received the same exposure to the learning procedure as the mice used for acetylcholine determinations.
- 2) Control Group for the Trained Mice- Sleeping times of this control group (19 mice) were determined simultaneous to those of the trained group
- 3) Shock-Stressed Group- These mice (total of 14) received the same stressing treatment as the group used for acetylcholine measurements.
- 4) Control Group for The Stressed Mice- Sleeping times were determined for this group (total of 28) concomitantly with those of the stressed mice.

In order to obtain some idea of the state of biological activity in the trained and stressed mice, the duration of barbiturate sleeping time in these mice was used. Driever and Bousquet (1965) has found that stress causes a decrease in hexobarbital sleeping time. They attributed this effect of stress to an alteration of the rate of drug metabolism. Furthermore, since sleeping times could not be altered in adrenalectomized or hypophy-

sectomized animals, this alteration of drug metabolism was suggested to be due to an activation of the pituitary-adrenal axis (Bousquet, et al, 1965). Therefore, hexobarbital sleeping times give an indirect indication of the degree of pituitary-adrenal activation.

Hexobarbital sleeping times were determined according to a method described by Garriott, et al, (1967). Seventy-five mg/kg hexobarbital was injected i.p. The time between the loss and regaining of the righting reflex was defined as the sleeping time.

Hexobarbital was injected immediately after the last shock episode or training session and the sleeping time was determined. As mentioned before, the hour long training and shocking period made only one experimental animal available per hour. Therefore, sleeping times had to be determined throughout the day. Since sleeping times were found to vary between the morning and afternoon, controls had to be run with every experimental mouse. In this research barbiturate sleeping times of two control, naive mice were always determined along with each experimental animal.

Statistical evaluations were made of the significance of the differences between the mean sleeping times of the trained group and its control and of the stressed group and its control. The unpaired Student-t was used (Batson, 1956).

#### c. Extraction And Bioassay Of Acetylcholine

Acetylcholine concentrations of brain parts were determined in 3 groups of M.m.CFL:

- 1) Trained Group
- 2) Shock-Stressed Group
- 3) Naive Control Group

The method of eserinated-acid-saline extraction used here was described in chapter III.

Acetylcholine was bioassayed using the guinea pig terminal ileum in the presence of antagonists of histamine and serotonin, namely, pyrilamine and morphine-dibenzylamine, respectively. This procedure has been detailed previously (confer Chapter III).

## C. Results

### a. The Effects Of Learning And Shock-Stress On Regional Brain

#### Concentrations Of Acetylcholine

The acetylcholine concentrations (mean  $\pm$  SE, microgram/gram brain tissue) in different brain parts immediately after learning an avoidance-conditioned procedure are shown on table 3. These values are compared with the acetylcholine levels of naive controls and expressed as the % change from control. This is expressed graphically in figure 9. The telencephalic (T) acetylcholine of the trained group was 3% lower, the midbrain-diencephalon (M-D) was 9% higher and the pons-medulla (P-M) 1% higher than that of the corresponding brain regions of naive (unshocked) control. None of these minor differences were statistically significant (figure 9). Determinations of cerebellar (C) acetylcholine were also attempted but the bioassays were not satisfactory. It was possible, however, to derive some information which indicated that the C extract of trained mice does not seem to contain more acetylcholine than that of the control mice.

Shock-stress treatment, in which the mice were not allowed to escape or avoid the shock, was found to have a selective effect on T acetylcholine concentrations (table 3 and figure 9). The T acetylcholine of the shock-stressed mice was 25% greater than that of the control group. This was a statistically significant increase ( $P < 0.05$ ). The 8% increase in M-D and the 10% decrease in P-M were not significant. Even when the acetylcholine values of the shock-stressed group are compared with those of the trained group, the T acetylcholine of the former group still represents a significant change. The M-D and P-M values are still not significantly different. Determinations of C levels of acetylcholine of the shock-stressed

Experimental Groups	Number of Ach Determinations*	Regional Brain Ach, gamma/gram $\pm$ SE		
		T <sup>+</sup>	M-D <sup>++</sup>	P-M <sup>+++</sup>
Naive Control	5	0.91 $\pm$ .03	1.25 $\pm$ .10	1.14 $\pm$ .12
Post-Learning	5	0.88 $\pm$ .08	1.36 $\pm$ .13	1.15 $\pm$ .12
% Change From Control		-3	+9	+1
Post-Shock-Stress	4	1.14 $\pm$ .08	1.35 $\pm$ .13	1.03 $\pm$ .08
% Change From Control		+25**	+8	-10

Table 3. The effect of learning and shock-stress on ach concentrations (mean  $\pm$  SE, micrograms/gram) in 3 brain regions of adult, male Mus musculus CF1 mice.

+ Telencephalon  
 ++ Midbrain-Diencephalon  
 +++ Pons-Medulla

\* 5 mice were used per determination  
 \*\* statistically significant



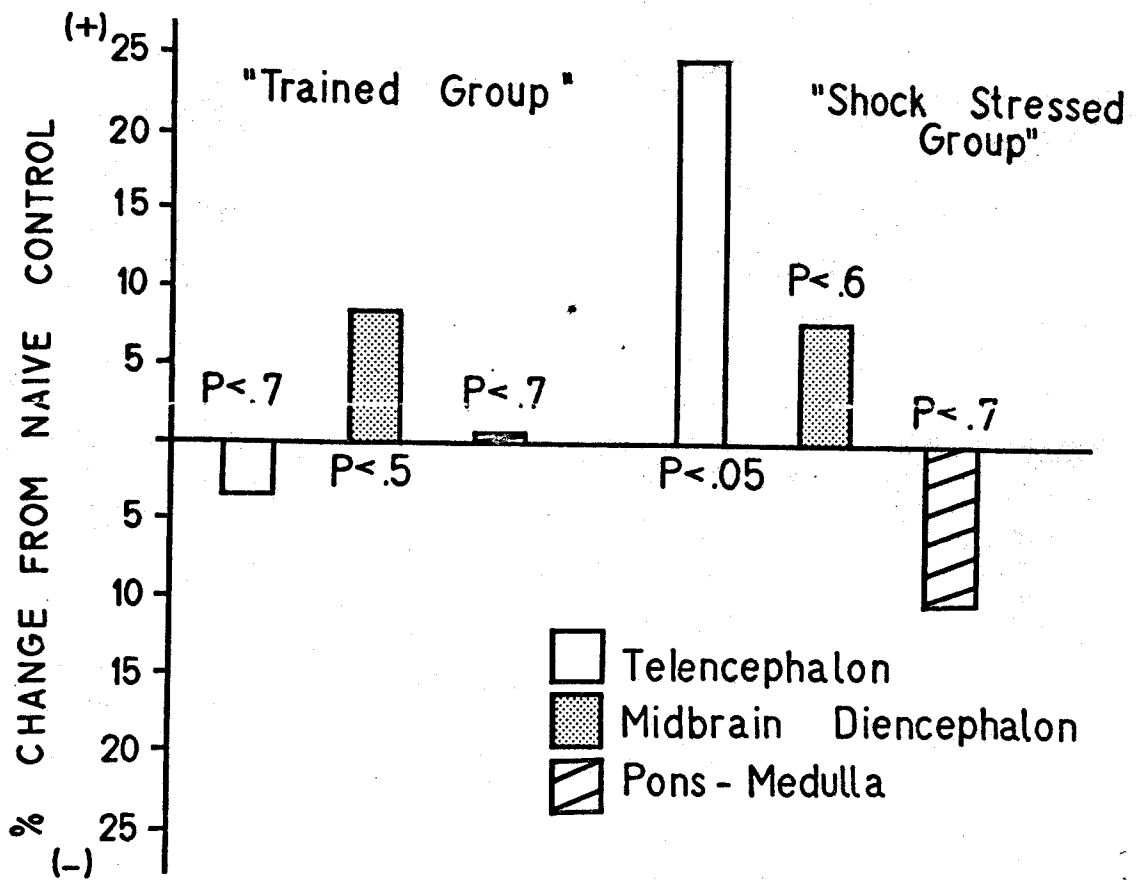


Figure 9. Percent change from naive, control, each concentrations in three brain regions of Trained and Shock-Stressed Mus musculus CF1 adult, male mice. P values are given for % changes of each brain part in both experimental groups.

group were again unsatisfactory, but there was some indication that the C acetylcholine of the shock-stressed mice is greater than that of either the control or trained groups. None of these experimental procedures differentially affected weights of brain parts.

**b. Stress-Associated Behavior**

Initially, both the trained and the stressed groups of mice responded strongly to the shock by jumping, squeaking and running. After each termination of the shock, the mice generally sniffed about, examining all aspects of the chamber in which they were present. Gradually, as the number of test sessions progressed, the trained group still responded in a similar qualitative and quantitative fashion. However, the stressed mice deviated notably from their earlier mode of response, thereby distinguishing them from the trained group. The stressed mice were observed to show more "freezing" behavior, almost catatonic in nature. This is uncharacteristic of normal Mus (Scudder, et al, 1969). After repeated shock, this stressed group now exhibited a markedly attenuated response of minimum jumping and running and slight vocalization. During the intertest intervals, the stressed mice replaced the earlier sniffing and exploratory behavior with behavioral withdrawal. Although these mice were quiet, they were not sleeping.

**c. The Effect Of Scopolamine On Stress-Induced Behavior**

The series of behaviors associated with an insolvable stress situation was found to be reproduced by using a simplified shock box. Mice placed in this box and shocked at repeated intervals demonstrated the initial maximum responses which then gradually reverted into the minimum reactions, freezing-catatonic posturing and quiet withdrawal.

The effect of an anticholinergic drug on this stress-induced behavior

was found to be rather marked. This preliminary study has indicated that 15 minutes after receiving an i.p. injection of 2mg/kg scopolamine HBr, the shock-stressed mice seldom exhibited the freezing, catatonic posturing or the quiet withdrawal. Rather, these mice displayed a high degree of exploratory behavior during the intershock interval. That this effect was reversible was shown by the reappearance of the freezing and withdrawal behaviors upon testing on the following day. The attenuated response to the shock itself was not interfered with by this dose of scopolamine once tolerance or habituation to the shock had been established. However, when scopolamine was given to an unshocked, naive animal, the development of such tolerance was delayed.

d. Effects Of Learning and Shock-Stress On Hexobarbital Sleeping Time

In the face of the behavioral withdrawal exhibited by the stressed mice but not by the trained animals, it was of interest to determine whether hexobarbital sleeping time (75mg/kg, i.p.) is differentially affected by shock-stress treatment but not by avoidance-conditioning. Compared with naive, control mice, the group trained in the climbing screen apparatus had a 1.4% lower sleeping time ( $P > 0.5$ ). Contrariwise, mice that had undergone shock-stress in the climbing screen, exhibited a 34.8% shorter sleeping time than controls. The P value for this difference was less than 0.1, being slightly less than a statistically significant difference of 0.05. There does, however, appear to be a tendency for an insolvable stress situation to cause a reduction in hexobarbital sleeping time compared to controls. Furthermore, no alteration in duration of sleeping time is present in mice which have been allowed to avoid or escape from the stressful situation.

As a corollary, hexobarbital (75mg/kg, i.p.) sleeping times for

M.m. SCl adult, male mice were found to be much longer in the morning (before 12:00 noon), 23 minutes, than in the afternoon (after 12:00noon), 16 minutes. Morning sleeping times averaged 41.5% longer than afternoon sleeping times.

#### D. Discussion

The acetylcholine concentrations in each of the 3 brain parts of the trained CFl mice reflect the state of the central cholinergic system, after a learned behavior has been successfully achieved, namely avoidance of a mild electric shock. Comparison with the acetylcholine levels of similar mice not exposed to such a training procedure, reveals that there has been no detectable, lasting alteration of the cholinergic system as result of training. This confirms similar findings of Toru, et al (1966) on rats. This lack of a detectable change in cholinergic activity is open to several interpretations. One possibility, of course, is that the cholinergic system plays no part in the learning process. This, however, seems to be a rather remote possibility in view of the mounting evidence in favor of cholinergic participation (Longo, 1966; Reeves, 1966; and see Introduction of this chapter). An alternative is that the cholinergic system functions only during acquisition of the proper response possibly to inhibit "wrong" responses (Carlton, 1963). Upon successfully responding, increased connectivity of the minimal number of neural circuits will occur, thereby strengthening the activation of this proper neural circuit (Roberts, 1966) and decreasing the need for the functioning of the inhibitory cholinergic system. This might be the condition of the mice in this experiment at the time of sacrifice. They had learned the problem and cholinergic activity was again normal. Another possibility is that the cholinergic system, complexed with other central neurochemical systems, serves as a structural component of those neural circuits involved in forming the behavioral response itself (Deutsch and Lutzky, 1967). As such, the cholinergic system should not be detectably (at least by the methods presently available) altered, since

only a minor group of cholinergic circuits would be involved. As a fourth suggestion, both the inhibition of non-rewarded responses as well as establishing the proper neural circuits themselves could be functions of the cholinergic system. None of the literature cited in the Introduction of this chapter really settles this problem as to how the cholinergic system functions in the process of learning. Prior experimental approaches have merely demonstrated a dependence of learning on the cholinergic activity. The ideal approach would be to magnify and prolong the events occurring during the acquisition or attempted acquisition of a learned response. Possibly, this is, in effect, what occurs when an animal is placed in an insolvable situation.

When an animal is placed in an experimental situation in which it must learn a correct response in order, for example, to avoid a noxious stimulus, it will display an array of behaviors in an attempt to adapt to that stimulus (Fuller, 1964; Roberts, 1966; and Scott and Fredericson, 1951). The experimenter may make the situation solvable or insolvable for the animal. Without prior knowledge or experience the animal will not be able to distinguish the 2 situations and, consequently, will respond initially in an identical fashion in both cases. That this occurs was substantiated in the present experiment as well as in previous work (Brown-Grant, et al, 1954; and Mallov and Witt, 1961). In the solvable situation the animal will, if capable, successfully learn the response. There is no logical reason to attribute the animal in the insolvable situation with an ability to "realize", at a certain point, the hopelessness of continued effort to solve its learning problem and intentionally to cease active attempts to solve the problem. (This however may be an automatic, physiological endpoint as will be discussed

subsequently). This animal should, therefore, continue to activate the physiological or neuronal mechanisms involved in learning the "correct" response thereby prolonging the learning process.

The "stressed" group of mice was placed in such an insolvable situation. Theoretically, then, the state of the biochemical systems in these mice should represent an accentuation of the state of biochemical activity during the normal period of acquisition or learning of a correct behavioral response. Of the three brain regions studied, only the telencephalon showed a significant change in acetylcholine concentration compared to naive (or trained) mice. This 25% increase in acetylcholine implicated a cholinergic mechanism, specifically restricted to the telencephalon, in the process of learning a correct response. This clearly substantiated most of the earlier reports (see Introduction of this chapter) claiming a cholinergic component in learning. However, this change in acetylcholine levels of "stressed" animals is contrary to the negative findings of Bowers, et al (1966) and Toru, et al (1966) but supports the report by Krause, et al (1964) that the forebrain cholinergic system is involved in emotional excitation. This work is also in keeping with the conclusion of Aprison, et al (1968) that a telencephalic cholinergic system is operative in the maintenance of behavioral excitation.

Classically, an increase in brain acetylcholine levels has been indicative of depressed cholinergic activity (Giarman and Pepeu, 1962; and Toru, et al, 1966). However, as explained in the Introduction to Section II of this thesis, valid exceptions to this rule are possible, for example, when prolonged activation occurs, increased recoverable acetylcholine can result. Several indications imply the increased telencephalic acetylcholine found in

the "stressed" mice is actually representative of increased activity. The marked reduction in hexobarbital sleeping times found to occur, here, for the "stressed" mice is an indirect indication of an activation of the pituitary-adrenal axis (Bousquet, et al, 1965; and Driever and Bousquet, 1965). The fact that a central cholinergic mechanism has been found to be intimately involved in release of ACTH (Hedge and Smelik, 1968) indicates a central cholinergic activation. Furthermore, the marked modification of the behaviors of the "stressed" mice by scopolamine, reported in this work, again strongly imply an active cholinergic system. A similar finding was reported by Ruther, et al (1966) using imipramine, which also has anticholinergic effects (Toman, 1963). Other reports in the literature attribute behaviors similar to those observed in the "stressed" mice used here as being due to a hyperactive cholinergic system (Delini-Stula, 1968; and Zetler, 1968). A hyperactive cholinergic system is also consistent with the finding of an increased metabolism of the central serotonergic (Eleftheriou and Church, 1968; Liberson, et al, 1964; and Welch and Welch, 1968) and adrenergic (Weiss, et al, 1961) systems after exposure to the stress of an insolvable situation.

Therefore, the results of this experiment are consistent with the theory of a general activation of the cholinergic system in learning to either inhibit non-rewarded responses and/or activate new increasingly complex behavioral patterns. These results do not however, support the possibility, mentioned earlier, that the cholinergic system functions only to establish the proper neuronal circuits. However, this possibility is by no means ruled out if the cholinergic system also serves to inhibit non-rewarded, or stimulate new, behavioral responses. According to Carlton (1963) the mechanism for inhibiting non-rewarded behavior is identical to that



responsible for habituation. This mechanism consists of an elaborate negative feedback system under the direction of the cortex (Roberts, 1966). Rosvola and Mishkin (1961) have attributed this property of suppressing inappropriate responses to the reticular system, thereby suggesting this to be the elaborate negative feedback system. Since Collier and Mitchell (1966-a and b and 1967) have been able to show that the ascending reticular system, permeating the cortex, is cholinergic in nature, the telencephalic cholinergic element, which in this thesis was suggested to be activated during learning, could well be the reticular negative feedback system controlling behavioral output. The final element in this negative feedback loop is the tonic, directive influence of the cortex on the reticular function demonstrated by Adey, et al (1957) and Bures, et al (1963). Under appropriate cortical direction, the reticular system is capable of either an inhibitory or facilitatory effect on incoming stimuli (Magoun, 1963; and Roberts, 1966) as well on ongoing behaviors (Carlton, 1963; Konorski, 1961; Magoun, 1963; Miles, 1964; and Rosvola and Mishkin, 1961). Therefore, the cholinergic reticular system is capable of effecting habituation to incoming stimuli, under the direction of the cortex (or other higher centers) as well as of carrying out the selection of an appropriate behavioral response, again, under the specific direction of the higher centers. In this respect one can appreciate the vital role played by the cholinergic system in habituation and other forms of learning.

In the Introduction of this chapter, the following questions were posed: "If the animal is not allowed to modify the shock, how long will the animal continue to test behavioral solutions?" and "Is the physiological make-up of the organism such that behavioral defensive maneuvers cease after a time?" The mice in both the "trained" and "stressed" groups responded to

the mild electric shock with typical alarm and defense responses — jumping, defecating, urinating, and increasing exploratory activity. The trained group gradually learned to avoid the shock but every time it did receive the shock, it responded in a similar way throughout. However, the group of mice receiving this stress in an inescapable situation gradually attenuated their responses. This attenuation was similarly observed by Brown-Grant, et al, 1954; and Mallov and Witt (1961). Besides the decreased reaction to the shock, the animals were characterized by variable periods of freezing or catatonia, depressed exploratory activity, apparent withdrawal or depression, remaining quietly awake, and a decreased responsiveness to stimuli. These observations were similarly found by other investigators (Grinker, 1966; Holmberg, 1954; Khan, et al, 1964-a and b; Lutz, 1967; Mallov and Witt, 1961; and Ruther, et al, 1966). It was obvious that these "stressed" animals were no longer making an effort to escape or avoid the shocking stimulus.

During these stress-associated behaviors, the telencephalic acetylcholine of these animals was 25% higher than normal. Evidence, cited earlier in this discussion, indicates this increased acetylcholine to represent an increased cholinergic activation. Furthermore, that this increased cholinergic activity was related to the appearance of at least some of these stress induced behaviors was evident by the reversal of the freezing, catatonic, and apparent depressant behaviors upon the administration of scopolamine. Similar reports are cited in the literature (Ruther, et al, 1966). Therefore, the appearance of these behaviors seems to result automatically from the continual increase in cholinergic activation during attempted learning. The catatonic and freezing behaviors indicated this heightened cholinergic activity. These behaviors have been previously associated with increased

cholinergic function (Naruse, et al, 1960; Zetler, 1968; and see Section I of this thesis). The withdrawal and apparent depression which followed can be explained as follows. Possibly, at a certain point of telencephalic cholinergic activation (associated with a specific level of serotonergic and adrenergic activity) the negative feedback system reached a sufficient level of activity to depress either sensory input or responsiveness to sensory input, this, behaviorally, would be expressed as depression and withdrawal. Increased biological activation was demonstrated for the "stressed" mice by their decreased hexobarbital sleeping times. Furthermore, EEG studies on stressed animals clearly demonstrated such animals to be in a state of physiological arousal (Freedman, et al, 1966). Therefore, in spite of their behavioral appearance, these animals are in a state of heightened activity. Alternatively, the increased cholinergic activity could have surpassed its optimum level of activation necessary for maximum behavioral performance (still maintaining neural activity), thereby resulting in a functional behavioral depression and withdrawal. The present experiment does not distinguish between these two possibilities.

Therefore, unsuccessful attempts to adapt to stress result in increased central cholinergic activation. Continual increase in reticular negative feedback cholinergic activity ultimately results in reduced response to sensory input objectively seen as behavioral depression and withdrawal, and accompanied by occasional catatonic freezing and depressed exploratory behavior. It was not determined in the present experiment whether this increase in telencephalic acetylcholine was the greatest increase that would occur even in the presence of longer stress. Furthermore, it is not known just how long this increased cholinergic activity is maintained in the presence of

continual stress.

Despite the fact that these stress-associated responses are partially effective in reducing the input of the noxious stimulus, they are generally considered maladaptive and neurotic behaviors in that they do not satisfy the needs of the organism (Wolpe, 1966). Similar responses have been found in humans (Grinker, 1966) exposed to prolonged stress. These patients appear withdrawn and depressed, while their degree of biological activity has been shown to be very high (Board, et al, 1956). Such neurotic responses in both humans and animals have been shown to resemble learned behaviors (Wolpe, 1966) in every respect except one -- their extraordinary resistance to extinction. The similarity of both human and animal neurosis and the fact that they are learned implies the presence of a hyperactive cholinergic system in both. Speculatively, since a learning process is involved, the minimum number of neural circuits will be consolidated to yield the learned response. Accordingly, the negative feedback cholinergic system would be expected to decrease in activity as the neurotic response becomes better learned. However, as mentioned previously it is possible for the cholinergic system to participate in the consolidated neuronal circuits. (Such participation could result in varying degrees of cholinergic activity). Circumstantial evidence indicates that this might be the case. The neurotic response will reoccur with every presentation of the stressing stimulus (or in association with the environment of the stress) with extraordinary persistence (Wolpe, 1966).

A release of ACTH is associated with continuous stressing events (Sacher, et al, 1967). Furthermore, ACTH is known to prevent extinction of a learned behavior (Bohus and DeWied, 1966). This could account for the persistence of the neurotic response. The implication of the cholinergic

system in the consolidated neuronal circuits is strengthened by the fact that ACTH release is intimately related to a cholinergic mechanism (Hedge and Smelik, 1968) and that acetylcholine synthesis in vivo is greatly facilitated by the presence of ACTH (Torda and Wolff, 1952-b).

Therefore, it is concluded that the process of adapting in the most effective manner to a noxious stimulus involves a continually increasing cholinergic activation (in conjunction with other biochemical systems). Initially an automatic physiological process of habituation is enforced, which has been suggested to rely on the cholinergic system. If the habituation process is unable to cope with the noxious stimulus, an increasing activation of the cholinergic system induces the appearance of a series of behavioral sets. If each of these behaviors is unsuccessful in modifying the threatening stimuli, it is inhibited and altered or incorporated into another more complex behavior. Eventually the cholinergic system reaches a degree of activation at which it is able to modify physiologically the sensory input. However, at this level of activation some additional effects occur: catatonia, "freezing" and depression of exploration. The modification of sensory input is not selective for the noxious sensory input but involves several sensory modalities and results in behavioral withdrawal and depression. This latter physiological modification may be interpreted to be an automatic, ultimate defense maneuver consequent upon the inability of behavioral varieties to remove the noxious stimulus.

At any point along this continuum of events learning and consolidation may occur. The one essential requirement is the appearance of an effective response under the circumstances. The determination of the most satisfactory response is probably an integrated function of the entire brain. Once the

best response is determined, its repetition induces consolidation of the proper neuronal circuits and a reduction in cholinergic negative feedback mechanisms.

It is concluded that the cholinergic component involved in trial and error effects control over neuronal circuits primarily in the telencephalic region of the brain.

CHAPTER VIII

EFFECT OF ISOLATION ON BRAIN ACETYLCHOLINE

OF MUS MUSCULUS SCI

## A. INTRODUCTION

There are several recognized types of fighting behavior (Karczmar and Scudder, 1968; and Scott and Fredericson, 1951). Only isolation-induced aggression will be discussed in this chapter. Aggressive fighting in mice is distinguished from the other forms of aggression on the basis of several parameters. Scott and Fredericson (1951) have described it as non-competitive, in the sense that it is not object-oriented as to the goal of the fighting. It can be maximally induced, and reproducibly so, simply by isolating the appropriate strain of adult Mus for a period of 3 weeks (DaVanzo, et al, 1966; Suchowsky, et al, 1967; and Yen, et al, 1959). The importance of the hormonal state of the animal is indicated in the fact that isolation will induce aggression only in the male animal (Valzelli and Garattini, 1967) and then only if the hormonal state of the male is functioning properly (Sigg, et al, 1966). The latter authors discovered that an intact pituitary-gonadal axis is essential to the formation of isolate aggression. Suchowsky, et al (1967) similarly found that depression of androgenic hormones and/or introduction of estrogenic hormones was deleterious to the induction of aggressiveness in adult mice. The reports of Bronson and Desjardins (1968) have shown that the development of aggressiveness in the adult is highly dependent upon the proper establishment of neural connections early in life, possibly during intra-uterine life (Abbatiello, 1968). The proper neural connections are part of the process of sexual differentiation, determined genetically and controlled hormonally (Abbatiello, 1968 and 1969; Bronson and Desjardins, 1968). In keeping with the apparent necessity of a hormonal component in the development of agonistic behavior, Scott and Fredericson (1951) hypothesized that an endocrine center, responsible for the physiological reactions of aggressiveness,



was located in the pituitary body. They further suggested a second, nervous center to exist in the hypothalamus.

Isolation changes other behavioral parameters as well as inducing aggression. Yen, et al (1959) noticed that isolate mice grow hyperexcitable, and more responsive to tactile and aural stimulation. Exaggerated jumping, head-twitching and hindleg scratching motions were observed when the mice were touched. Horripilation (hair literally stands on end over most of the body), ferocity and extreme aggressiveness were also noted. Confirmation of such hyperactivity and hyperirritability induced by isolation was reported by Weltman, et al (1962) and also by Agrawal, et al (1967). In general, isolated mice present a picture of increased stimulus sensitivity. The suggestion that long-term isolation enhances excitability is supported by the finding that mice isolated prior to treatment are more sensitive to amphetamine than pre-grouped animals (Welch and Welch, 1966). It is important to realize that, regardless of pretreatment, amphetamine toxicity is increased, if the mice are placed in a grouped environment after injection, while toxicity is lower in mice isolated after injection (Chernov, et al, 1966; Weiss, et al, 1961; and Welch and Welch, 1966).

Scott and Fredericson (1951) proposed a rather interesting theoretical mechanism to explain the induction of aggression by isolation. This mechanism could also account for the increased excitability and responsiveness to stimuli which occurs during isolation (vide supra). Their proposal is based on the partial removal of habituation to incoming stimuli. According to these investigators, the presentation of a strange male to the isolated mouse triggers off an aggressive episode in the isolated animal, because some sensation is generated by the strange intruder, a tactile,

auditory, visual or olfactory stimulus which is transmitted to the brain of the isolated mouse and triggers the aggressive behavior. These authors postulate that the neural connections required for the aggressive behaviors are genetically determined and established very early in life. Even young mice have the capacity to exhibit aggressiveness but such behavior necessitates the presence of a proper hormonal state. Such a proper state of hormonal balance does not establish itself until the mouse reaches approximately 25-30 days of age. Consequently, if the animal is raised with its litter-mates, for the first 25-30 days of life, the mouse is continually exposed to the same tactile, auditory, visual or olfactory sensation which would normally trigger off an aggressive episode in an isolated animal, but since the young mouse is incapable of responding, repetitive exposure to such, now, non-significant stimuli results in the activation of the natural mechanism of habituation, as postulated by Roberts (1966). Scott and Fredericson, therefore, have suggested that mice raised in an aggregate environment become habituated to the stimuli which in adulthood would have generated an aggressive episode. Since only repetitive stimuli are habituated to (Roberts, 1966), maintenance of habituation to the aggression-generating stimuli necessitates constant confrontation of the animal with such stimuli, namely other mice.

Scott and Fredericson (1951) further proposed that isolation for a sufficient period of time from the stimulation of other mice would serve to eliminate the habituation that had developed to the presence of other mice during the maturation period of the animal. Subsequent introduction of a strange male into the isolation cage would allow aggression-generating stimuli to act in the brain of the isolated animal to promote an attack. During the period of isolation, habituation to many sensory stimuli will have been

weakened; consequently, presentation of a mild sensory input results in an exaggerated response in the isolated animal, as has been demonstrated experimentally

Changes in organ weights have also been found to distinguish isolate from aggregate animals. The experiments of Rosenzweig, et al (1968) have shown specific differences in brain weight. Grouped rats raised in an highly stimulating environment have significantly larger amounts of cortical material than isolated, sensorily impoverished rats. However, little difference was detected between their subcortical weights. The increased cortical weight in aggregate rats was at least partially attributed to an increased rate of glial proliferation (Altman and Das, 1964). Zolman and Morimoto (1962) showed that such increased cortical weight is lost when the aggregate rats were subsequently isolated. A decrease in brain weight upon isolation has been confirmed by Geller, et al (1965).

Several reports have associated prolonged isolation with a decrease in adrenal weight and prolonged aggregation with an increase in adrenal weight (Giacolone, et al, 1968; and Welch and Welch, 1966). However, opposite changes were found in adrenal weights by Geller, et al (1965) and Sigg, et al (1966).

Changes in body weight have also been reported, increasing in aggregate and decreasing in isolate conditions (Geller, et al, 1965; and Giacalone, et al, 1968). These results also conflict with others; Rosenzweig, et al (1968) and Sigg, et al (1966) found isolate animals to weigh more than aggregates

The biochemical profile of isolated animals, described in the literature, is as follows. Norepinephrine (NE) brain levels have been found

to either increase (Geller, et al, 1965; and Richardson, unpublished data) or remain unchanged (DaVanzo, et al, 1966) in isolated animals. Welch and Welch (1964 and 1965) reported increased brain levels of NE and reduced turnover rate in isolated mice. Under similar conditions of isolation, serotonin (5-HT) brain levels either decreased (Welch and Welch, 1968) or remained unaltered (DaVanzo, et al, 1966; Geller, et al, 1965; and Giacolone, et al, 1968). Very discrete changes in 5-hydroxy-indole-acetic acid (5-HIAA), the major degradatory metabolite of 5-HT, have been detected. Welch and Welch (1968) found a decrease in whole brain 5-HIAA and Giacolone, et al (1968) identified this decrease to be specifically localized to the midbrain-diencephalon region of the brain. The importance of the serotonergic system in this brain region to isolation-induced aggression was indicated by the fact that Giacolone and his group found no change in 5-HIAA in female mice, which do not become aggressive. Based upon a study of 5-HT turnover rates and upon the decrease in 5-HIAA, Welch and Welch (1968) and Giacolone, et al (1968) agree that prolonged isolation induces a decrease in 5-HT metabolism.

The importance of the midbrain-diencephalon brain region in isolation induced aggression was indicated by the select change in 5-HT metabolism in this part of the brain of only aggressive mice (Giacolone, et al, 1968). The major changes in brain amino acids during isolation occur in the midbrain-diencephalic area (Agrawal, et al, 1967), thereby further implicating this brain region in isolation induced aggression. Perhaps this supports Scott and Fredericson's (1951) suggestion of an aggression-controlling center located somewhere in the hypothalamic region (a major constituent of the diencephalon).

Other changes in amino acids (Agrawal, et al, 1967) have also been found to occur throughout the brain in response to isolation. Marcucci, et al

(1968) uncovered a significant decrease in N-acetyl-L-aspartic acid (NAA) throughout the brains (except for the corpora quadrigemina) of male mice isolated for approximately three weeks. However, as with 5-HT changes (Giacolone, et al, 1968), no changes in NAA were found in isolated females. The previously described contention that isolated animals are hyperexcitable gains further support from this decrease in NAA brain levels, since low NAA levels in mice brains are correlated with increased nervous activity (Buniatian, et al, 1965).

Other characteristics of isolated animals are that they contain relatively lower blood levels of corticosteroids than aggregates (DaVanzo, et al, 1966; and Geller, et al, 1965) and that blood levels of free fatty acids (FFA) are proportionately lower in isolate than aggregate mice (Giacolone, et al, 1968).

In view of the fact that one of the most effective pharmacological agents to reduce isolate aggression in mice is an anticholinergic, scopolamine (DaVanzo, et al, 1966), it is surprising that so little effort has been made to clarify the role played by the cholinergic system in isolation-induced aggression. While inferences can be made from the reduced cholinesterase during isolation as to the level of brain acetylcholine (Rosenzweig, et al, 1968; and Zolman and Morimoto, 1962), no actual determinations of acetylcholine concentrations in aggressive, isolated mice have been reported. Such information is the raison d'etre of this chapter. Levels of acetylcholine will be presented for three brain regions of mice made aggressive by isolation for at least 3 weeks. These are compared with those of the same brain areas in aggregated, unaggressive mice.

## B. MATERIALS AND METHODS

### a. Animals

Throughout the experiment adult, male Mus musculus SC1 mice were used. They were obtained from Scientific Small Animals Farm, Illinois, at approximately 60 days of age.

### b. Experimental Design

The 60 day old male SC1 mice were divided into two groups. The mice in the "isolate" group (total of 25 mice) were housed individually in plastic cages (7.5 x 5.5 x 3.5 inches). Those in the "aggregate" group (total of 20 mice) were housed in groups of 15 to 20 in large cages (18 x 10 x 6 inches). All mice received food and water ad libitum. Isolation in these cages, with a minimum of handling, was maintained for at least 21 days, which is ample time for aggression to have developed (DaVanzo, et al, 1966; Suchowsky, et al, 1967; and Yen, et al, 1959). This method for inducing aggression is in compliance with the standardized method of isolation-induced aggression which was compiled during the International Symposium on Aggressive Behavior (Valzelli, et al, 1968). All mice were kept in a constant temperature chamber.

After the appropriate period of isolation, the average age of the mice was 88 days. The mice isolated or grouped were placed in the operating room about 30 minutes before sacrificing. No contact between aggressive mice was allowed. Each mouse was removed from its cage. weighed and sacrificed immediately by decapitation. The sequence in which aggregated or isolated mice were used to determine acetylcholine was randomly varied. In this way the 2 experimental groups compared more closely in terms of age and the influence of uncontrolled variables was minimized. In order to account for any possible circadian effects, all mice were sacrificed at approximately

10:00 A.M.

The regional concentrations of acetylcholine in the isolated mice were compared to those of the aggregated mice. Statistical analysis of any differences between the two groups was carried out using the Student-t test (Batson, 1956). In addition, the weights of the brain regions of the two groups of mice, as well as their body-weights, were similarly compared and statistically analyzed.

c. Extraction And Bioassay

The brains were divided into three regions, the telencephalon, midbrain-diencephalon and pons-medulla, and acetylcholine was extracted using the eserinizid-acid-saline method. The bioassay of acetylcholine was carried out using the guinea pig terminal ileum (confer Chapter III for details).

### C. RESULTS

#### a. Effects Of Isolation On Regional Brain Acetylcholine Concentrations

Isolation of male, adult Mus musculus SC1 for 21 days was found to result in a small (10%), but very significant ( $P < .02$ ) drop in M-D acetylcholine concentration compared to control aggregated mice (Table 4 and Figure 10). The acetylcholine changes in T and P-M brain regions did not approach significance.

#### b. Effects Of Isolation On Body Weight And Regional Brain Weights

Isolated mice were found to differ from aggregated animals not only in their M-D acetylcholine concentrations, but also in their body weights and regional brain weights (Table 5).

Isolated SC1 mice were distinguished from aggregated animals by being 10% lighter in body weight (a highly significant difference at  $P < 0.01$ ). In addition, both the T and P-M of the isolates weighed 7% less than the same brain parts in aggregated mice. Both of these latter differences were found to be statistically significant. However, the M-D weights of both groups of mice were virtually identical.

An analysis of the changes in brain part weights and in acetylcholine concentrations induced by isolation presents an interesting possibility (confer Tables 4 and 5). The significant decrease in brain weight of both T and P-M of isolated mice, coupled with the fact that neither of these regions demonstrated a significant change in acetylcholine concentration (i.e. micrograms acetylcholine/gram tissue), implies that the absolute amount of acetylcholine may have decreased in both brain regions in proportion to the decrease in weight of these brain parts. A calculation of the total amount of acetylcholine per brain part for each determination of acetylcholine,



Experimental Group	Regional Brain Ach, Gamma/Gram $\pm$ SE		
	T <sup>+</sup>	MD+++	PM+++
Aggregates	0.76 $\pm$ .04 (20/4)*	1.08 $\pm$ .01 (20/4)	0.76 $\pm$ .13 (10/2)
Isolates	0.83 $\pm$ .04 (25/5)	0.97 $\pm$ .03 (25/5)	0.74 $\pm$ .04 (25/5)
% Change From Aggregate	+9	-10**	-3

Table 4. Ach concentrations (mean  $\pm$  SE, micrograms/gram) in three brain parts of isolated and aggregated, adult, male Mus musculus SC1 mice.

+ Telencephalon  
 ++ Midbrain-Diencephalon  
 +++ Pons-Medulla

\* Total number of mice used/number of determinations  
 \*\* Statistically significant

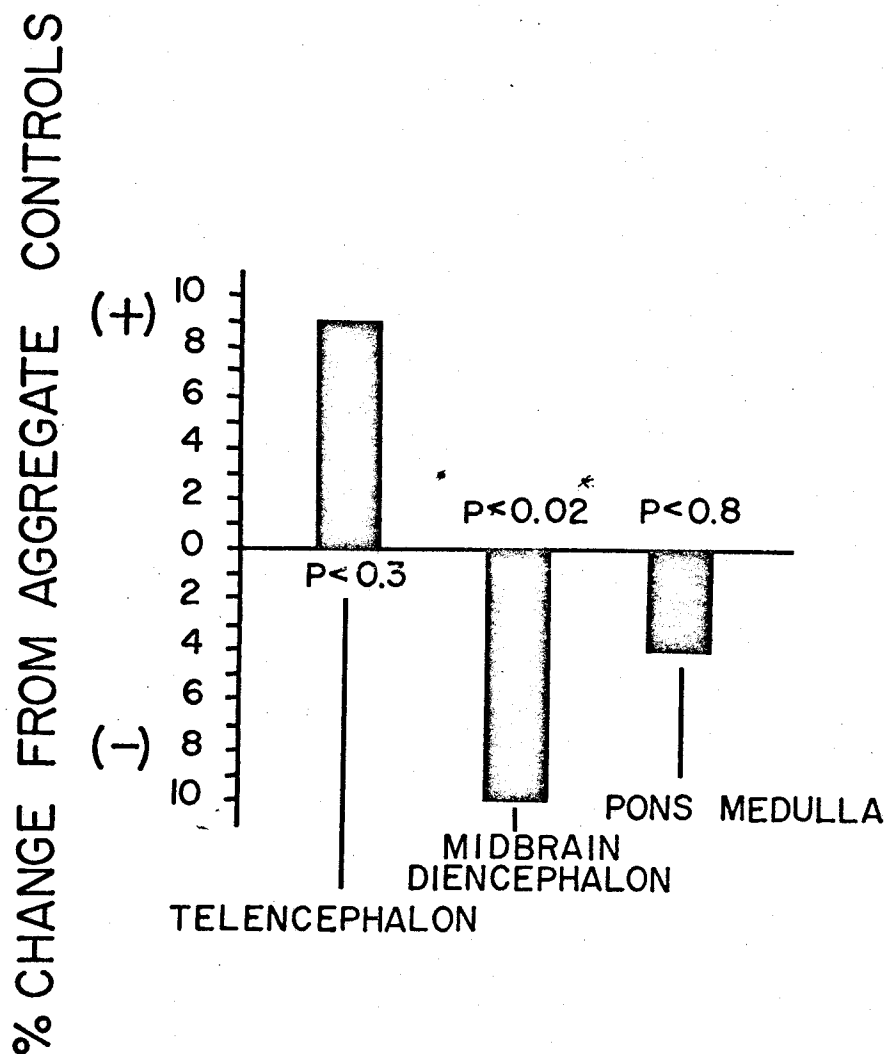


Figure 10. Percent change from aggregate, control, ach concentrations in three brain regions of isolated, adult, male, Mus musculus SC1 mice. P values are given for % changes of each brain part.

Experimental Group	Body Weight, grams $\pm$ SE	Weights of Brain Parts, grams $\pm$ SE		
		T <sup>+</sup>	MD <sup>++</sup>	PM <sup>+++</sup>
Aggregates	42.0 $\pm$ 0.9 (20)*	0.2946 $\pm$ 0.004 (20)	0.0920 $\pm$ .005 (20)	0.0740 $\pm$ .001 (20)
Isolates	38.0 $\pm$ 0.7 (25)	0.2731 $\pm$ 0.003 (25)	0.0909 $\pm$ 0.002 (25)	0.0689 $\pm$ 0.002 (25)
% Change From Aggregate	-10** P 0.01	-7** P 0.01	-1 P 0.9	-7** P .05

Table 5. Average weights (mean  $\pm$  SE, grams) of three brain parts and average body weights (mean  $\pm$  SE, grams) of isolated and aggregated adult, male Mus musculus SC1 mice.

+ Telencephalon  
 ++ Midbrain-Diencephalon  
 +++ Pons-Medulla

\* Total number of mice used  
 \*\* Statistically significant

however, revealed no significant differences between the T and P-M of isolate and aggregate mice. Therefore, no substantiation for this possibility of a change in total acetylcholine in T or P-M was found. But one significant difference in total acetylcholine was found. The M-D of isolated mice contains a 13% lower amount of acetylcholine.

#### D. DISCUSSION

There has been a tendency on the part of some investigators to regard isolation, or sensory deprivation, as a stressful event (Giacolone, et al, 1968; and Weltman, et al, 1962). This attitude has been based on reports that sensory deprivation can, under certain circumstances, generate disruptive effects on personality and mental functioning resembling a schizophrenic-like syndrome in humans (Goldberger and Holt, 1958; Wexler, et al, 1958). Similarly, behavioral abnormalities have been noted in rats raised in an impoverished environment (Rosenzweig, et al, 1960). The induction of aggression in isolated rodents (Yen, et al, 1959) has been suggested to be abnormal behavior indicative of stress (Giacolone, et al, 1968).

However, this popular belief is unsubstantiated (at least for animals) by most of the available biochemical and physiological data on isolated animals, including the data presented in this experiment. To the contrary, many have shown that the aggregated animal is much more stressed than the isolated animal. In brief, stress is characterized by increased activity of the serotonergic (Welch and Welch, 1968) adrenergic (Weiss, et al, 1961) and cholinergic (see Chapter VII of this thesis) systems. Additionally, plasma free fatty acids (FFA), pituitary-adrenal activation and urinary catecholamines are markedly increased in animals under stress (Mallov and Witt, 1961; Michalova, et al, 1966 and Moore, 1966). Associated with increased adrenal activation is hypertrophy of the adrenal glands during prolonged states of stress (Welch and Welch, 1966). Furthermore, amphetamine toxicity is greatly increased when animals are exposed to stress (Weiss, et al, 1961). A more detailed discussion of stress is found in Chapter VII of

this thesis.

Welch and Welch (1968) described isolated animals, unlike aggregated mice, to have depressed serotonergic functioning, which was localized to the region of the midbrain-diencephalon by Giacolone, et al, (1968). The lower corticosteroid plasma level (Da Vanzo, et al, 1966), as well as the small amount of plasma FFA (Giacolone, et al, 1968) in isolated animals implies a similar depressed functioning of the adrenergic system. Several investigators have associated the depressed corticosteroid plasma level with decreased adrenal weights in isolated animals (Giacolone, et al, 1968; Welch and Welch, 1966). However, this latter finding is questioned by the results of Sigg, et al, (1966) and Geller, et al (1965).

Even though many of the biochemical and physiological parameters are decreased in isolated mice, they have been shown to be more sensitive to central stimulants than are grouped mice (Chernov, et al, 1966; Weiss, et al, 1961; and Welch and Welch, 1966). However, while preisolation does enhance such sensitivity to stimulants, the state of "aggregation" itself was shown to be more stressing than isolation in that amphetamine toxicity is much greater in mice grouped after injection compared to mice isolated after injection.

Comparison of these parameters between stressed and isolated animals clearly differentiates an isolated from a stressed or grouped animal (the latter 2 resembling each other closely).

A consideration of the cholinergic component in isolated mice was taken up in the present thesis. Compared to aggregate mice, isolation was found to result in a small but significant decrease of acetylcholine concentration in the midbrain-diencephalon region of the brain, while no

significant change was noted in telencephalic or pons-medulla acetylcholine.

That this decreased level was accompanied by a reduced activity of the cholinergic system within the same brain region is indicated by Geller, et al (1965) and Rosenzweig, et al (1960) who reported decreased subcortical ACHE activity in isolated impoverished animals. La Torre (1968) repeated these environmental studies using mice. He similarly reported a decrease in ACHE in the subcortex (corresponding to the midbrain-diencephalon used here) but no significant change in cortical ACHE. This supports the findings presented here of lower acetylcholine in the midbrain-diencephalon but no change in the telencephalon. A decreased cholinergic activity is also consonant with the proposed depressed serotonergic and adrenergic systems of isolate animals (Da Vanzo, et al, 1966; and Giacolone, et al, 1968). In view of the hyperactive cholinergic\*system associated with stress (Chapter VII of this thesis) and high emotionality (Section 1 of this thesis), the lowered central cholinergic activity in isolated mice opposes the proposition that these animals are stressed.

The induction of aggressiveness by isolation has been suggested to involve, specifically, the midbrain-diencephalon region of the brain (Giacolone, et al, 1968). These authors found serotonin metabolism to be depressed only in this region and only in animals that become aggressive, eg. not in female mice. Furthermore, changes in central amino acids occurred primarily in this brain region after isolation (Agrawal, et al, 1967). The importance of this same brain region in isolation-induced aggression gains further support from the present findings. The decrease in cholinergic activity was localized to the midbrain-diencephalon region of the brain. Therefore, aggression, induced by isolation, occurs in association with a

certain degree of cholinergic inactivation in the midbrain-diencephalon. This seems contrary to fact that a most potent antiaggressive agent effectively reduces central cholinergic activity, namely scopolamine (DaVanzo, et al, 1966). However, as will be demonstrated in Chapter IX of this thesis, the antiaggressive effect of scopolamine can be better correlated with its telencephalic effects.

Several reports have shown that prolonged isolation not only enhances aggressiveness (Welch and Welch, 1966) but also general excitability (Adams, et al, 1966; and Welch and Welch, 1966). Buniatian, et al (1965) and Adams, et al, (1966) referred to such changes as a reduction in resistances and defensiveness. Such behavioral excitability is consistent with the postulate of Scott and Fredericson (1951) that isolation results in a regression of habituation to sensory input. As a learned behavior (Roberts, 1966 and Ungar, 1967) habituation is subject to extinction and requires periodic reinforcement. In the absence of habituation, sensory stimuli (normally restricted by the negative feedback of habituation) would have easier access to the central nervous system. Since Carlton (1963) has postulated that habituation is due to a cholinergic mechanism. the reduction of cholinergic activity in the present experiment supports Scott and Fredericson's (1951) contention of a regression of habituation during isolation. The present results further suggests that the process of habituation, referred to, involves the cholinergic system within the midbrain-diencephalic region of the brain. While the telencephalic and pons-medullary acetylcholine levels are not altered significantly, these brain regions do undergo a significant reduction in weight during isolation. However, there is no change in weight of the midbrain-diencephalon. These findings more



clearly localize the changes in brain weight reported by Geller, et al (1965) and Rosenzweig, et al (1960) and confirm and extend the findings of LaTorre (1968). One explanation for the change in brain weight was suggested by Altman and Das (1964) who found the number of glial cells to be higher in aggregated than isolated animals. An indication that a reduction in glial cells does indeed accompany the reduced telencephalic brain weight is supplied by the work of LaTorre (1968). This author found a reduction of pseudo-ChE in the cortical regions (telencephalon) of the brains of isolated mice. No change in pseudo-ChE was found in the subcortex (midbrain-diencephalon). This finding is significant since pseudo-ChE is associated primarily with non-nervous, glial tissue in the brain (Hebb and Krnjevic, 1962; Holmstedt and Toschi 1959; Koelle, 1954 and 1963; and Torack and Barrnett, 1962). According to Roberts (1966) a decrease in glial tissue suggests a decrease in neural growth or activity. However, the cholinergic system was shown here not to be measurably affected (neither total acetylcholine nor concentrations of acetylcholine was significantly altered in either of these two brain parts). Similarly, Giacalone, et al (1968) presented evidence indicating the serotonergic system is not affected in these regions. However, specific regional changes of other suggested transmitter systems, e.g. adrenergic, have not yet been investigated in isolated animals. A decrease in general neuronal activity in the telencephalon or in the pons-medulla is not consistent with the finding that isolated animals are in fact in an increased state of activity (Welch and Welch, 1966). The proposed decrease in glial cells could, however, indicate a regression of synaptic connectivities (Roberts, 1966). Speculatively, neuronal connections for select behavioral responses (stemming from both telencephalic and pons-medullary sites), learned prior to isolation,

may regress during the period of isolation when these neuronal connections are not being stimulated to function. It would be interesting to test the level of performance of a learned behavior after a certain period of isolation.

Therefore, from this work it can be concluded that the induction of aggressive behavior by isolation is associated with a minor but significant depression of the midbrain-diencephalic cholinergic system. This can be interpreted to reflect a regression of a certain degree of habituation to incoming stimuli (Scott and Fredericson, 1951). Previously restricted stimulation from an intruding mouse is now capable of generating an aggressive attack, which is postulated to be an innate behavior of the rodent (op.cit).

CHAPTER IX

REGIONAL ALTERATIONS OF BRAIN ACETYLCHOLINE IN ISOLATED

MUS MUSCULUS SCI BY SCOPOLAMINE HYDROBROMIDE

## A. INTRODUCTION

Cholinolytics, to the exclusion of other types of pharmacological agents, are most effective in reducing aggression. ( DaVanzo, et al, 1966; Janssen, et al, 1960; and Karczmar and Scudder, 1968). Few plausible mechanisms to explain the antiaggressive efficacy of anticholinergics, especially scopolamine, have been proposed. One theory of some merit was suggested by Longo (1966). He proposed that cholinolytics, eg. scopolamine, disrupt the sensorial triggering of the aggressive behavior. Longo based this theory on the conclusions made by Meyers and Domino (1964) that scopolamine interferes with the central organization of sensory information.

However, much information is available as to the general effects of cholinolytics on brain activity and function. Cholinolytics are known to cause a dissociation between EEG and behavior (Bradley, 1968). Normally, a fast frequency, low voltage EEG pattern is associated with alertness and a slow, high voltage pattern with sleep. However, administration of an anticholinergic causes a slow, high voltage EEG pattern but also behavioral changes that tend towards increased excitation (Bradley, 1968; Giarman and Pepeu, 1962; and Wikler, 1952). It has been amply demonstrated that anticholinergics reduce the sensitivity of cortical cholinceptive cells to synaptic excitation and to iontophoretically applied acetylcholine (Krnjevic and Phillis, 1963 a and b). Furthermore, the EEG arousal effects of applied acetylcholine and of cholinesterase inhibitors are antagonized upon administration of cholinolytic drugs (Bradley and Elkes, 1957; Miller, et al, 1940; and Szerb, 1964). Therefore, the depression of the arousal or alert pattern of the EEG by cholinolytics is explainable on the basis of inhibition of the postulated cholinergic arousal system (Rinaldi and

Himiwich, 1955). Collier and Mitchell (1966 a and b, and 1967) substantiated the presence and functioning of such an arousal system by showing the stimulation of one of 2 ascending cholinergic reticular systems producing an EEG arousal mediated by an increased output of acetylcholine.

The induced behavioral excitation is, however, more difficult to explain. Varying theories have been proposed. An inhibition of central cholinergic mechanisms may "release" non-cholinergic systems (White and Rudolph, 1968). This theory was based on Carlton's (1963) postulate that acetylcholine partakes in depressing the appearance of non-rewarded behavior. The antagonism of this function would, consequently, allow the expression of inappropriate or random behavioral activity. Another possible explanation, suggested by Meyers and Domino (1964) is that scopolamine interferes with the organization of sensory information that regulates the reaction of the animals. This would result in disorganized, random behavioral responses. A third possibility is that cholinolytics may inhibit the process of habituation to incoming stimuli, which habituation is highly dependent upon the adequate functioning of the cholinergic system (Carlton, 1968). This would allow an uninhibited input of sensory stimuli to which the animal would be constantly responding.

In spite of their ability to produce some behavioral excitation, cholinolytics are classed primarily as sedatives or central depressants (Goodman and Gilman, 1965). This is in part based on the fact that their pharmacological profile resembles depressants in the sense that they facilitate barbiturate sleeping time (Giarman and Pepeu, 1962; and Mensch and DeJongh, 1958).

Attempting to define the brain sites at which anticholinergics

act, Rinaldi and Himwich (1955) and White and Rudolph (1968) reported electrophysiological data suggesting these drugs to have both a cortical and midbrain site of action. This was supported by the report of Veit and Vogt (1935) that scopolamine enters the midbrain. The finding by Fink and Urban (1966) that cholinolytics altered acetylcholine levels in both cerebral and mesencephalic brain regions added additional support to the suggested sites of action. However, these results were questioned by the findings of Giarman and Pepeu (1964) and Beani, et al (1964) that anticholinergics depress acetylcholine concentrations only in the cerebral hemispheres and not in subcortical regions. The results presented in this thesis may help to clarify this point.

This decrease in acetylcholine concentrations by anticholinergics, such as atropine and scopolamine, is a well known but, again, inexplicable phenomenon (Beani, et al, 1964; Crossland and Slater, 1968; Fink and Urban, 1966; and Giarman and Pepeu, 1962 and 1964). Beani, et al (1964) and Giarman and Pepeu (1964) have shown this decrease to be attributable to some mechanism other than inhibition of choline acetylase or enhancement of cholinesterase activity. By collecting from perfused brain regions, Mitchell (1963) and Szerb (1964) were able to demonstrate that the decrease in brain acetylcholine concentration is attributable to an increased rate of release of acetylcholine from the nerve terminals. One proposal made by Giarman and Pepeu (1964) considers the anticholinergic to exert some influence on the storage sites of acetylcholine. This contention receives support from the work of Creese and Taylor (1965) who found atropine to decrease the uptake of labelled carbachol by isolated brain tissue. Furthermore, Crossland and Slater (1968) studied the select effects of cholinolytics on the specific

forms of acetylcholine, namely the labile or free form and the stabile or bound form. They found that the decrease in whole brain acetylcholine levels is due to a select decrease in the stabile or bound form of acetylcholine, further supporting the proposal made by Giarman and Pepeu (1964).

An alternative explanation of how anticholinergics induce an increased output (i.e. decreased brain levels) of acetylcholine stems from Roberts' (1966) suggestion that immediately following depolarization a direct feedback inhibitor might be released to control the presynaptic neuronal activity. Increasing postsynaptic activity would result in increased negative feedback activity inhibiting or depressing further presynaptic activity. An anticholinergic drug, which blocks postsynaptic activity, would reduce negative feedback thereby facilitating release of more excitatory transmitter (acetylcholine) from the presynaptic neuron. A similar explanation, based on a compensatory feedback mechanism, was described by Nyback, et al (1967) for the increased activity in monoaminergic neurons following adrenergic receptor blockade.

Most of the pharmacological and neurochemical effects of scopolamine have usually been studied in aggregated or unaggressive animals. Before an explanation can be offered to explain how scopolamine antagonizes isolation-induced aggression, its pharmacological and neurochemical effects will have to be verified in isolated aggressive animals. At this laboratory, Richardson, (unpublished data), has found scopolamine to reduce whole brain 5-HT and increase norepinephrine and dopamine concentrations in isolated, laboratory mice. Similar studies using cholinolytics in either isolated or aggregate animals, have not previously been reported.

The research of this chapter is devoted to studying the effect of

an antiaggressive dose of scopolamine on acetylcholine concentrations in three distinct brain parts of isolate mice. A dose response curve was obtained at this laboratory (Scudder, unpublished data) for the antiaggressive effects of scopolamine. At 15 minutes after 2 mg/kg i.p., isolation-induced aggression was depressed to 50% of the control level; at 1 hour after the same dose, aggression was further depressed to 20%. The acetylcholine level at these two time intervals after this dosage was determined to see whether the temporal increase of the antiaggressive effect of 2 mg/kg scopolamine can be time related to specific, regional changes in brain acetylcholine levels.



## B. MATERIALS AND METHODS

### a. Animals

Mus musculus SC1 adult, male mice were used throughout. They were obtained from Scientific Small Animals Farm, Illinois at approximately 60 days of age. All animals were kept in a constant environmental chamber and received food and water ad libitum.

### b. Experimental Design

The 60 day old, male mice were isolated individually. This method of inducing aggression by isolation was described in Chapter VIII of this thesis.

The following study was run on SC1 mice isolated in this manner for more than three weeks. There were four experimental groups of isolated mice:

1. A saline control group (20 mice) injected with physiological saline i.p., 15 minutes before sacrifice. Previous studies at this laboratory have shown these mice to exhibit approximately 80% control aggression (Scudder, unpublished data).

2. An experimental group (20 mice) injected with 2 mg/kg scopolamine hydrobromide, i.p., 15 minutes before sacrificing; this dose of scopolamine at 15 minutes post-injection has been previously shown to reduce aggression to 50% (Scudder, unpublished data).

3. A saline control (10 mice) injected with a physiological saline, i.p., 1 hour before sacrifice. Mice treated in this way were found to exhibit approximately 80% control aggression (Scudder, unpublished data).

4. A scopolamine experimental group (15 mice) injected with 2 mg/kg of drug, i.p., and sacrificed 1 hour later; the aggression of this group

was previously shown to be reduced to 20% of the control value (Scudder, unpublished data).

No significant differences in acetylcholine levels were found between the 2 saline controls. Their values have been tabulated together as one saline control to obtain a more accurate estimate of control acetylcholine. These saline control acetylcholine values were used to compare the regional brain acetylcholine concentrations of both scopolamine treated groups. Any differences between the control and experimental groups were evaluated statistically for levels of significance using the Student-t test (Batson, 1956).

Circadian effects were eliminated by sacrificing all the animals at 10:00 A.M. The sequence in which determinations of acetylcholine levels were made in the different experimental groups was randomized in order to minimize the influence of other possible variables, such as age at the time of sacrifice.

#### c. Extraction And Bioassay

Excision of brain tissue, extraction of acetylcholine and the guinea pig ileum bioassay have been described in Section I of this thesis (confer Materials and Methods - Chapter III).

Since the guinea pig ileum is responsive to the muscarinic action of acetylcholine, and since cholinolytic drugs are characterized as being muscarinic inhibitors (Goodman and Gilman, 1965; and Turner, 1965), the possibility existed that some of the scopolamine, administered to the mouse, might be recovered in the brain extract and, subsequently, interfere with an accurate quantitation of the tissue acetylcholine. This possibility was considered by Giarman and Pepeu (1964). They found the administered drug,

scopolamine, not to interfere with the subsequent bioassay using the guinea pig ileum. This conclusion was based on the following 2 experimental results: 1) quantitation of a tissue extract from an animal that had received scopolamine, using both the guinea pig ileum and frog rectus, the latter being insensitive to cholinolytics, yielded similar acetylcholine values; and 2) acetylcholine added to the extract obtained from animals that had received scopolamine was found to have equivalent potency on the guinea pig ileum compared to an equal amount of acetylcholine added to the ileum in the absence of extract. In spite of this finding by Giarman and Pepeu (1964) that the guinea pig ileum bioassay is accurate for scopolamine-treated animals, the present author conducted a similar check. On several occasions during this experiment, a known amount of acetylcholine was added to the unknown tissue extract, after the original bioassay was finished. This extract was again assayed against the original standard. The percent recovery of the added acetylcholine was determined and used to indicate the degree of interference due to the presence of any scopolamine in the tissue extract. This experimental check was carried out several times both with the 15 and 60 minute scopolamine groups, as well as with the saline control. All three brain region extracts were checked in this manner. There was no difference in percent recovery of acetylcholine between either of the scopolamine group extracts or the saline control extracts. Therefore, the conclusion of Giarman and Pepeu (1964) was confirmed; scopolamine, administered to the mice at a dose of 2 mg/kg, did not interfere with the subsequent bioassay of acetylcholine using guinea pig ileum.

### C. RESULTS

Scopolamine had a profound effect on regional brain acetylcholine concentrations in isolated, Mus musculus SC1 mice (Table 6 and Figure 11). Within 15 minutes of an i.p. injection of 2 mg/kg scopolamine, T acetylcholine levels dropped precipitously to 51% ( $P < 0.001$ ) of control levels. The M-D acetylcholine was decreased by 37% ( $P < 0.02$ ). There was a 14% decrease in P-M acetylcholine which was not significant ( $P < 0.2$ ). One hour after the same dose, these effects were somewhat moderated. Acetylcholine concentrations of T was still 37% lower than saline control ( $P < 0.01$ ), while the acetylcholine of M-D was now only 18% lower than control ( $P < 0.2$ ) and P-M was down 7% ( $P < 0.7$ ); the latter two changes were not statistically significant. Therefore, in summary, a sharp reduction in brain acetylcholine levels occurs initially in T and M-D regions but, subsequently, the M-D levels approach control values, while the T acetylcholine remains considerably lower than control.

Experimental Treatment	Duration of Treatment	Regional Brain Ach, Gamma/Gram $\pm$ SE		
		T <sup>+</sup>	MD <sup>++</sup>	PM <sup>+++</sup>
Saline, i.p.	*	0.94 $\pm$ .05 (30/6)**	1.16 $\pm$ .08 (25/5)	0.87 $\pm$ .05 (30/6)
Scopolamine HBr 2 mg/kg, i.p.	15 minutes	0.46 $\pm$ .02 (20/4)	0.73 $\pm$ .11 (20/4)	0.75 $\pm$ .07 (20/4)
% change from saline control		-51***	-37***	-14
Scopolamine HBr 2 mg/kg, i.p.	1 hour	0.59 $\pm$ .07 (15/3)	0.95 $\pm$ .09 (15/3)	0.81 $\pm$ .11 (15/3)
% change from saline control		-37***	-18	-7

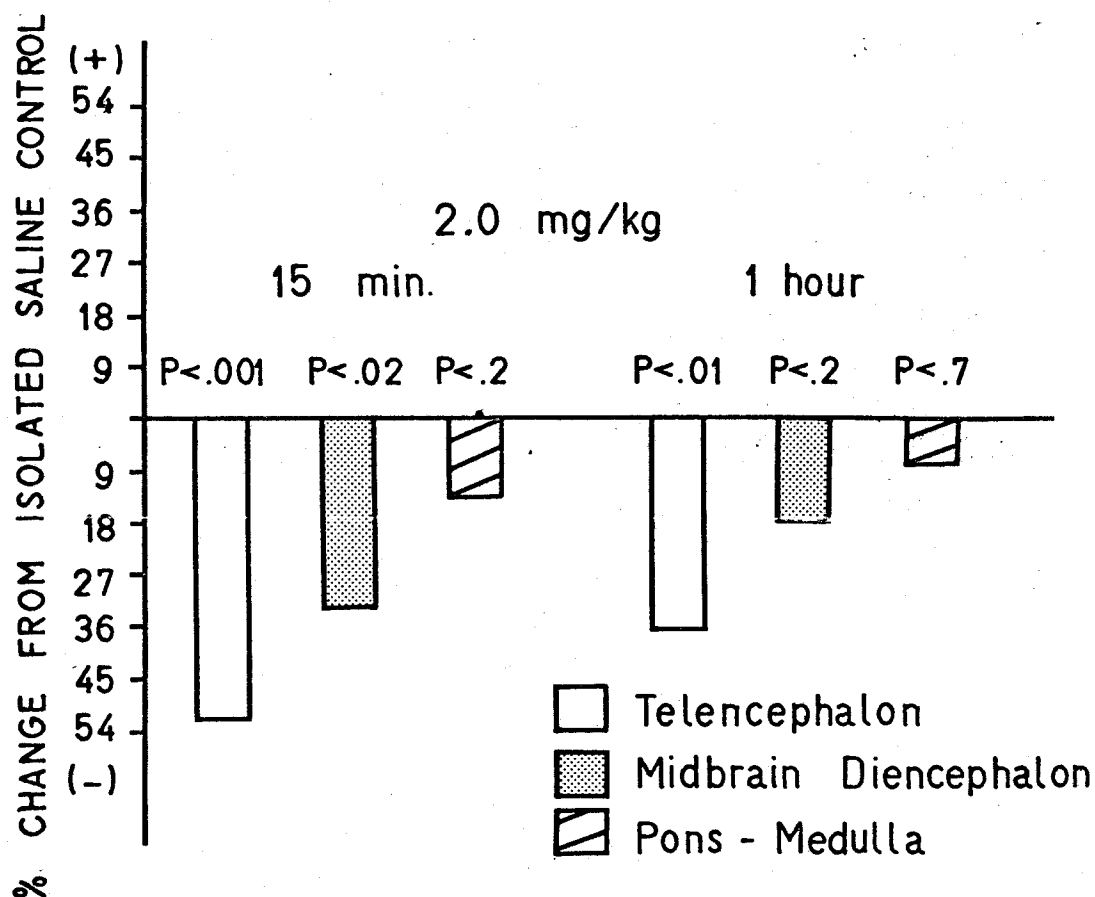
Table 6. Effect of 2 mg/kg (i.p.) scopolamine HBr, at 15 minutes and 1 hour post injection, on ach concentrations (mean  $\pm$  SE, micrograms/gram) in three brain regions of isolated, adult, male Mus musculus SC1 mice.

+ Telencephalon  
 ++ Midbrain-Diencephalon  
 +++ Pons-Medulla

\* Saline values at 15 minute and 1 hour post injection were combined, since no significant differences were found between them in these experiments.

\*\* Total number of mice used/number of determinations

\*\*\* Statistically significant



**Figure 11.** Percent change from isolate, saline, control, each concentration in three brain regions of isolated, adult, male *Mus musculus* SCl mice 15 minutes and 1 hour after a 2 mg/kg dose of scopolamine HBr (i.p.). P values are given for % changes of each brain part in both experimental groups.

#### D. DISCUSSION

The presented results are in agreement with the reported effects of cholinolytics in aggregated, non-aggressive animals (Beani, et al, 1964; Crossland and Slater, 1968; Fink and Urban, 1966; and Giarman and Pepeu, 1962 and 1964) and show that scopolamine (2 mg/kg, i.p.) decreases acetylcholine concentrations in isolated, aggressive mice. More specifically acetylcholine was initially (15 minutes post injection) depressed in the telencephalic and midbrain-diencephalic regions of the brain but not in the pons-medulla. This contrasts with the localization of effect to only the cerebral brain region by Beani, et al (1964) and Giarman and Pepeu (1964). On the other hand, the present results compare favorably with the findings of Fink and Urban (1966) and support the suggestion that anticholinergics have both a cortical and midbrain site of action (Rinaldi and Himwich, 1955; and White and Rudolph, 1968).

The maximum effect of scopolamine to decrease brain acetylcholine occurred 15 minutes after injection. By 1 hour post injection, the midbrain-diencephalon acetylcholine levels approximated control values; the reduction of telencephalic acetylcholine was also attenuated but concentrations were still significantly below control. Comparison of this time sequence of regional acetylcholine reduction with the antiaggressive effects of scopolamine (2 mg/kg) at 15 minutes and 1 hour after injection shows that the peak antiaggressive effect was found to occur 1 hour post injection at which time aggression was only 20% of the control (Scudder, unpublished data). At 15 minutes after receiving this dose of scopolamine, aggressive attacks were depressed to only 50% of control. Therefore, the antiaggressive effect of scopolamine is better related to a reduction of telencephalic acetylcholine,

rather than to that of midbrain-diencephalic acetylcholine. In view of the involvement of the cholinergic system in the midbrain-diencephalon in the mechanism of induction of aggression in isolated animals (refer to Chapter VIII of this thesis), the present data indicates that scopolamine does not exert its primary influence on this mechanism whereby aggression is induced by isolation. Rather, it affects some cholinergic component involved in the expression of this type of aggressive behavior, apparently within the telencephalic region of the brain. One possibility is that it retards cortical reception or integration of the aggression-generating sensory input within the cortex itself as postulated by Longo (1966). This suggestion is based on the fact that a cholinergic arousal system has been shown to exist (Collier and Mitchell, 1966-a and b and 1967; and Rinaldi and Himwich, 1955) and to be intimately related to the EEG pattern of arousal. Krnjevic and Phillis (1963-a) have shown cholinolytics to depress the sensitivity of cortical cholinceptive cells and Bradley (1968) described the depression of the arousal or alert pattern of the EEG.

Another possibility is that scopolamine interferes with the cholinergic mechanism of depressing inappropriate responses to a stimulus (Carlton, 1963). This assumes that cortical reception of the aggression-generating stimuli is not defective but that the behavioral response is inappropriate.

Other alternatives could be postulated including the possibility that scopolamine exerts its antiaggressive effect primarily in the midbrain-diencephalon or the pons-medulla but that its effect on acetylcholine is either not detectable by the techniques used in this thesis, or not related to its antagonism of aggression.



CHAPTER X

REGIONAL ALTERATIONS OF BRAIN ACETYLCHOLINE CONCENTRATIONS IN

MUS MUSCULUS CF-1 INDUCED BY METHIONINE SULFOXIMINE (MSI)

## A. INTRODUCTION

One of the first biochemical anomalies to be definitively associated with chronic human epilepsy was a disruption of the ability to bind acetylcholine in the epileptogenic focal brain tissue (Tower, 1955; and Tower and Elliott, 1953). The concomitant finding of increased cholinesterase activity (Tower, 1955; and Tower and Mc Eachern, 1949) was interpreted by Tower as a compensation for the increased release of free acetylcholine. The importance of the cholinergic system in convulsive activity has previously been suggested by Hyde, et al (1949), when they found that the convulsive activity of several, different chemically unrelated drugs was enhanced by anticholinesterases and other cholinomimetics. An actual participation of the cholinergic system in convulsions was further indicated by the decreased brain levels of acetylcholine immediately after drug or electrically-induced seizures (Richter and Crossland, 1949). Elliott, et al (1950) disproved the theory that this decrease in brain acetylcholine was due to an inhibition of synthesis by demonstrating an increased recovery of acetylcholine in brain perfusion chambers during the convulsive episodes. Richter and Crossland (1949) proposed that adequate brain levels of acetylcholine are necessary for seizure activity to occur. They suggested this to be a possible explanation for the increased frequency of seizures in epileptics during early anesthesia and sleep, when acetylcholine is expected to be high (Crossland and Merrick, 1954). This suggestion that an increased level of acetylcholine serves as a primer for convulsive activity has received considerable support since its conception. By pharmacologically altering the threshold, intensity or duration of electroshock convulsions and, subsequently, measuring acetylcholine levels, Takahashi, et al (1961) were able to relate seizure intensity as well as its duration

directly with brain acetylcholine levels. However, seizure threshold alteration was not found to be directly dependent upon the cholinergic system. The fact that young animals are less prone to convulse than older animals (Naruse, et al, 1960; and Schlesinger, et al, 1965) fits well with the postulated priming function of acetylcholine, because young animals contain significantly lower brain levels of acetylcholine than do older animals (Crossland, 1951; Crossland and Merrick, 1954; Feldberg, 1944-45; Naruse, et al, 1960; and Welch and Hyde, 1944).

Genetic studies have added additional support for the importance of acetylcholine in convulsive seizures. Using genetically determined convulsive strains of mice, Kurokawa, et al (1963), Naruse, et al (1960) and Takahashi, et al (1961) associated high acetylcholine brain levels with a predisposition to seizure. Correspondingly, Pryor (1968) found AChE activity to be highest in convulsive strains of mice. Naruse, et al (1960) specified the higher acetylcholine levels in convulsive strains to be due primarily to the bound or stabile form of acetylcholine. They attributed the onset of seizure to the conversion of the bound (stabile) to the free (labile) form. The reviews of Fink (1966) and Reeves (1966) present additional available data confirming the intimate involvement of the cholinergic system in the convulsive process.

More detailed investigations into the mechanism of convulsions on a chemical level required defining the conditions contributing to the conversion of bound to free acetylcholine. (Richter and Crossland (1949) postulated the need of additional factors besides adequate levels of acetylcholine for the generation of the convulsive episode. The genetic studies of Takahashi, et al (1961) supported this, in that acetylcholine levels were not found to correlate

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with the changes in seizure threshold but did correlate with seizure intensity and duration. Toman and Everett (1958) found that seizure threshold could be lowered (i.e. increased susceptibility to seizure) by decreasing brain levels of biogenic amines. Raising the amine levels would, alternatively, raise the threshold (i.e. decrease susceptibility). Ample substantiation of this effect on seizure threshold was offered by both genetic (Schlesinger, et al, 1965; and Scudder, et al, 1966) and pharmacological (Saito and Tokunaga, 1967; and Schlesinger, et al, 1968) studies. In addition, the property of biogenic amines of reducing seizure susceptibility was also associated with 5-HT (Schlesinger, et al, 1965 and 1968; and Scudder, et al, 1966) and gamma-aminobutyric acid (GABA) (Saito and Tokunaga, 1967; Schlesinger, et al, 1968; and Tada, 1963).

GABA and its related glutamic acid-glutamine system have been correlated with the cholinergic system on several occasions. In the genetic study of Naruse, et al (1960) seizure prone strains of mice had high levels of acetylcholine, as well as, low glutamine, glutamic acid and ammonia levels but high levels of GABA. Roberts (1960) interpreted the high levels of GABA (an inhibitory transmitter) to be in compensation for the increased acetylcholine concentrations (an excitatory transmitter). In addition, after Tower and Elliott (1953) revealed the decreased binding capacity of acetylcholine in human epileptogenic focal tissue, Tower (1955) subsequently reported a metabolic loss of glutamic acid which was not replenished. The finding of low glutamic acid and an association of convulsive activity with an hyperactive cholinergic system in both the epileptogenic tissue and the brains of the seizure prone mice strains might have been disregarded as coincidental, if it were not for the presentation of additional findings. These same

biochemical changes were found to be mimicked by anoxia and by a drug called methionine sulfoximine (MSI), both of which are associated with convulsive behavior (Tower, 1955; and Tower and Elliott, 1953). This therefore indicated the possibility of a basic relationship between the cholinergic system and the glutamic acid-glutamine - GABA system involved in the mechanism of convulsive activity.

As a convulsant drug, methionine sulfoximine has been shown to mimic human epilepsy more closely than any other known drug (Tower, 1955 and 1960; and Tower and Elliott, 1953). Administration of MSI to an animal results in a series of periodic convulsive seizures but only after a latency of several hours, depending upon dose and type of animal used (Harris, 1964; Lamar and Sellinger, 1965; and Sellinger and Rucker, 1966). The seizure response to MSI in animals takes the form of frantic running fits, associated with dilated pupils, piloerection and "fearful expression" (Wada, et al, 1967). These responses are occasionally accompanied by grand mal type seizures. No reports have described the state of the animal during the preconvulsive latency period. However, Harris (1964) has found that the EEG, several minutes after MSI was administered, showed increased cortical potentials, thereby differing from the later pattern signaling the onset of convulsions, when decreased potentials occurred. Therefore, the EEG data indicates the MSI treated animal to be in an opposite state shortly after drug administration as compared to the later stage of seizure.

A sample of the brain tissue of animals convulsed by MSI shows several distinct biochemical changes as compared to normal brain tissue. The suggestion that MSI interferes with acetylcholine binding (Elliott and Penfield, 1948) was borne out by the studies of Tower and Elliott (1953). Incubation of

brain tissue from MSI convulsed animals revealed that, while synthesis of acetylcholine was apparently normal, the mechanism of binding to form the stabile acetylcholine was impaired. The level of free or labile transmitter was, however, not affected. Mc Lennan and Elliott (1951) reported high concentrations of MSI to depress formation of free acetylcholine and low concentrations to, at least, facilitate the appearance of free acetylcholine. The ineffectiveness of MSI to alter the free acetylcholine levels in Tower and Elliott's (1953) experiments implies the concentration of this drug in brain tissue to be rather low. The biochemical lesion induced by MSI was interpreted to cause a continual release of acetylcholine, which, under proper conditions, could generate a seizure. Furthermore, this defective acetylcholine-binding mechanism, resulting from MSI administration, could be corrected by adding glutamine asparagine, methionine or glutamic acid to the incubation medium. These compounds had no obvious effect on normal tissue. Since anoxic tissue and human epileptogenic focal tissue exhibited the same binding defect, Tower and Elliott (op. cit.) tested the reversing ability of the above compounds on these tissues. Correction of the defect was achieved with glutamine or asparagine but not with glutamic acid or methionine additions. These results therefore indicate the possibility that MSI, anoxic or epileptic seizures commonly involved the cholinergic system, in association with a deficit of asparagine and glutamine. Even though glutamic acid was ineffective in reversing the binding defect, this amino acid seems to be central to all the sequel that occur in convulsions. Possibly this is because the 2 major metabolic pathways involving glutamic acid result in the synthesis of glutamine and aspartic acid (precursor to asparagine), respectively (Salganicoff and De Robertis, 1965; and White, et al, 1964).

To determine whether MSI influences the brain metabolism of this glutamic acid - glutamine system, Tower (1955) repeated the incubation study, this time measuring glutamic acid, glutamine and acetylcholine changes. The defect in acetylcholine-binding was reconfirmed. The second biochemical lesion found was a decrease or a metabolic loss of glutamic acid and, to a greater extent, glutamine. These effects on glutamic acid and glutamine were confirmed by Peters and Tower (1959) who additionally found MSI to cause a depression of tissue GABA levels. The restoration of glutamic acid and, to a lesser extent of GABA, could be accomplished by the addition of methionine or asparagine. However, the glutamine loss was not corrected. Therefore, the authors attributed the effects of MSI to an inhibition of glutamine synthesis from a reaction between glutamic acid and ammonia, catalyzed by glutamine synthetase. Inhibition of this enzyme had previously been suggested by Elliott, 1948). Since the measures employed by these authors to restore the low levels of glutamic acid and GABA were not successful in correcting the low glutamine levels, and since these same measures, namely methionine or asparagine addition, were effectively used to restore acetylcholine-binding in similar MSI convulsed animals (Tower and Elliott, 1953), Peters and Tower (1959) stated that the maintenance of acetylcholine-binding capacity was a function of glutamic acid or, less likely, GABA.

Subsequently, evidence had been presented showing MSI to irreversibly inhibit glutamine synthetase in vivo (De Robertis, et al, 1967; Lamar, 1968; and Lamar and Sellinger, 1965); but, since inhibition of this enzyme occurs some time before the onset of seizure and since subconvulsive doses of MSI also inhibit it to a maximum degree, Lamar (1968) proposed that seizure production is not dependent solely on the inhibition of this enzyme. An

antagonism of other enzymes participating in the glutamic acid-glutamine-GABA system has been shown. For example, convulsive doses of MSI depress the activities of: glutamine transferase (Lamar and Sellinger, 1965), which converts glutamine back to glutamic acid and ammonia; glutamic acid decarboxylase, which serves to form GABA from glutamic acid; aspartate-amino-transferase, which, to a minor extent, converts glutamate to aspartate; and alanine-amino-transferase, which catalyzes the conversion of alanine to glutamate and vice-versa (De Robertis, et al, 1967). De Robertis also found essentially no change in monoamine oxidase activity in MSI treated tissues but there were decreased brain levels of both norepinephrine and 5-HT. The MSI convulsion was shown to induce a small decrease in acetylcholine levels and a non-significant decrease in AChE activity. This finding of minor changes in the cholinergic components, together with the finding by Gershoff and Elvehjem (1951) that there was no difference in whole brain levels of acetylcholine of MSI convulsed animals compared to normals, led De Robertis, et al (1967) to disparage a cholinergic involvement in MSI induced convulsions. However, in view of the positive results of Elliott and Penfield (1948), Tower and Elliott (1953), Tower (1955), Peters and Tower (1959) and others, and since reports of the association of the cholinergic system with MSI are based on the use of tissue from convulsing animals in which state acetylcholine metabolism is known to be rapid (Richter and Crossland, 1949), perhaps the exclusion of the cholinergic system from consideration is an error. In fact, pharmacological evidence presented by Wada and Ikeda (1966) implicates a central role for acetylcholine in MSI induced seizures. They found that atropine markedly reduced the frequency of MSI induced seizure activity and that eserine facilitated such seizure activity. In view of this apparent



contradiction this question is investigated further in the experiments described in this chapter.

In summary of the above, the complex of biochemical effects associated with MSI induced convulsions indicate that glutamic acid, glutamine and GABA are depressed at the time of seizure. A malfunctioning binding mechanism for acetylcholine has also been considered. The low levels of either glutamic acid or GABA may be partly responsible for the depression of acetylcholine binding mechanisms. These events, with few exceptions, are based upon changes detected after or during the MSI induced convulsions. Only 2 reports in the literature have indicated the effect of MSI on any of the above biochemical parameters in subconvulsive doses or during the preconvulsive period. Lamar and Sellinger (1965) did a time study on the effect of convulsive doses of MSI on brain glutamine synthetase and glutamine transferase. Both enzymes were inhibited within 30 minutes after injection and this inhibition progressed in intensity reaching a maximum by 4 hours. Convulsions did not occur until 4-6 hours after injection. These same authors and Lamar (1968) found glutamine synthetase to be inhibited by subconvulsive doses of MSI to almost the same extent as convulsive doses. There are no reports on the levels or activities of the other neurochemical components, including acetylcholine, during the preconvulsive stage or after subconvulsive doses of MSI. In agreement with the statement made by Saito and Tokunaga (1967) that, convulsion should not be considered solely in terms of the ictal period, the research of this chapter will delineate the state of the cholinergic system in three brain regions during the preconvulsive stage of MSI-seizures. Additionally, a subconvulsive dose of MSI has been studied as to its effect on the brain acetylcholine levels in the same brain regions.

MSI was of particular interest to this author for another reason, aside from its seizure-inducing capacity. In spite of the evidence indicating MSI to be a convulsive drug releasing acetylcholine, Heath (1966) attributed this drug with an anticholinergic function and described it as promoting schizophrenic-like behavior in control subjects. He offered an explanation of this anticholinergic effect by postulating that MSI, an antimetabolite of methionine (Gershenovich, et al, 1963), interferes with the formation of acetylcholine by blocking the synthesis of choline in which methionine participates as 5-adenosylmethionine, a methyl donor (White, et al, 1964).

In addition, MSI has been found to alter certain behavioral parameters in mice at subconvulsive doses (25 and 50 mg/kg, i.p.) (Scudder, unpublished data). At the lower dose exploratory behavior was increased by 80%, stereotypy was depressed by about 77% and aggression was virtually abolished. Learning ability was somewhat increased by 15%. The 50 mg/kg dose similarly reduced aggression, but it also depressed exploration, stereotypy and learning. The effects of this higher dose were found to closely resemble the disruptive effects of a convulsive dose of MSI (100 mg/kg) during the preconvulsive period. The general depression of aggression and increased exploration at the 25 mg/kg dose is reminiscent of the effect of an anticholinergic agent (Bradley, 1968; Da Vanzo, et al, 1966; Giarman and Pepeu, 1962; and Wikler, 1952), thereby lending some support to Heath's (1966) suggestion that MSI exerts an anticholinergic effect.

The present experiment contributes information pertinent to this suggestion; if MSI does, in fact, inhibit acetylcholine synthesis, it should decrease acetylcholine levels before convulsive seizures have begun

and at subconvulsive doses.

## B. MATERIALS AND METHODS

### a. Animals

Determinations of brain acetylcholine levels were carried out using adult, male, Mus musculus CF1 mice that were approximately 79 days old and weighed about 33 grams. They were obtained from Carworth Farms, New Jersey.

Hexobarbital sleeping times were determined using adult, male, Mus musculus SC1 mice weighing approximately 33 grams.

The pilot study on toxicity of MS1 in young and old mice used white, laboratory, male mice obtained from Abrams Small Animals Farm (Illinois). Two groups of mice, differing in their ages, were used: one group was 50 days old, the other 90.

The pilot study with scopolamine was carried out using 62 day old CF1 mice.

All mice were housed in groups of 20-25 mice per cage in a constant environmental chamber. Food and water was supplied ad libitum.

### b. Experimental Design

#### i. Acetylcholine Study

The effect of MS1 on regional brain acetylcholine levels was determined using 2 doses (i.p.) of the drug: 100 mg/kg (a convulsive dose) and 25 mg/kg (a subconvulsive dose). The effects on acetylcholine levels of the convulsive dose were studied at 45 minutes and at four and one half hours after administration. In an attempt to distinguish the effects of a convulsive and a nonconvulsive dose of MS1 on brain acetylcholine, the lower dose was investigated only at four and one half hours post injection. Identically treated saline controls were run concomitantly. Therefore, three experimental groups of mice were used:

- 1) sacrificed four and one half hours after receiving 100 mg/kg MS1, i.p. (total of 24 mice) or physiological saline (total of 20 mice);
- 2) sacrificed four and one half hours after receiving 25 mg/kg MS1, i.p. (total of 20 mice) or physiological saline (total of 10 mice); and
- 3) sacrificed 45 minutes after receiving 100 mg/kg MS1, i.p. (total of 15 mice) or physiological saline (total of 10 mice).

Determinations of acetylcholine at each dose and time period were carried out as separate experiments.

All mice sacrificed four and one half hours after treatment were injected at 9:30 A.M. and allowed to remain in the operating room until the time of sacrifice at 2:00 P.M. Since the acetylcholine values for the four and one half hours saline controls of the 100 mg/kg MS1 experimental group and of the 25 mg/kg MS1 group did not differ significantly from each other, their acetylcholine values were averaged and used as a single four and one half hours control in order to more accurately estimate control values.

The mice to be sacrificed 45 minutes after receiving 100 mg/kg MS1 were injected at 1:15 P.M. and decapitated at 2:00 P.M. Identically treated saline controls were used to compare acetylcholine values.

The mean acetylcholine values of each brain part for each of the 3 experimental conditions were statistically compared with the appropriate saline controls and standard errors computed. The level of significance of the difference in acetylcholine between each experimental and control group was determined according to the method of Student-t (Batson, 1956).

ii. Determination of hexobarbital sleeping times in mice pretreated

with MS1

MS1 induced modifications of hexobarbital sleeping times were used to gauge the state of biological activity of the drugged mice. Sleeping times were determined in three experimental groups:

- 1) Four hours after 100 mg/kg, MS1;
- 2) Four hours after 25 mg/kg MS1; and
- 3) Sixty minutes after 100 mg/kg MS1.

Each group contained its own, identically treated saline controls.

Hexobarbital sleeping times were determined according to the method described by Garriott, et al (1967). Seventy-five mg/kg hexobarbital was injected i.p. and the time between the loss and regaining of the righting reflex was defined as the sleeping time.

A total of 27 mice were used for the determination of sleeping time in the first experimental group. Fifteen of these mice received 100 mg/kg, MS1, i.p. and 12 were injected with saline. Four hours later barbiturate sleeping times were determined for both groups.

The second experimental group consisted of a total of 38 mice. Nineteen were saline controls and 19 received 25 mg/kg MS1, i.p. Sleeping times were determined four hours later.

Thirty-five mice were used for the third group; 15 were controls and 20 were injected with 100 mg/kg, MS1, i.p. 60 minutes after treatment hexobarbital was given and sleeping times measured.

The percent of increased or decreased sleeping times compared to control was determined for each group. Any changes in sleeping time compared to saline control were statistically evaluated using the Student-t (Batson, 1956).

iii. Pilot studies considering the relationship of MS1 toxicity to age and scopolamine.

Toxicity and age

To test whether a convulsive dose of MS1 is more lethal in young or old animals, 100 mg/kg MS1 was given i.p. to 2 differently aged groups of mice. The percent mortality was determined 24 hours later. One group, consisting of 30 mice, was 50 days old and the second group of 30 mice was 90 days of age. Each group was subdivided into 6 sub-groups of 5 mice each. Each sub-group was placed in a separate cage. All of the mice were injected i.p. with 100 mg/kg MS1 at 10:30 A.M. The next day, 24 hours after injection the percent mortality values for each sub-group of 5 mice was computed, yielding a total of 6 determinations for each age group. The mean percent mortality was then calculated for each age group.

Effect of scopolamine on MS1 seizure and toxicity

Since MS1 involve an uninhibited release of acetylcholine (Tower, 1955) and since anticholinergics temper the seizure activity due to MS1 (Wada and Ikeda, 1966), the effect of scopolamine HBr, an anticholinergic, on MS1 convulsions, as well as toxicity, was studied. This pilot study used 3 groups of 5 mice each (the average age of the mice was 62 days):

Group 1) received 100 mg/kg MS1, i.p. and 5 injections of physiological saline every hour thereafter for 5 hours;

Group 2) received MS1 at a dose of 100 mg/kg with 20 mg/kg scopolamine HBr and, subsequently, received a 20 mg/kg dose of scopolamine every hour for 5 hours; and

Group 3) received 20 mg/kg scopolamine HBr every hour for 6 hours.

Any appearance of convulsive behavior was recorded and the percent

mortality of each group, 24 hours after treatment, was noted.

c. Extraction And Bioassay

Acetylcholine determinations were made on three brain regions, telencephalon (T), midbrain-diencephalon (MD) and pons-medulla (PM). These quantitations were made using brains from several experimental groups of CFl mice:

- 1) mice that had received either physiological saline or 100 mg/kg MS1, i.p., and sacrificed four and one half hours later;
- 2) mice injected with either 25 mg/kg, MS1 or physiological saline and sacrificed four and one half hours later; and
- 3) mice receiving either 100 mg/kg, MS1 or saline and subsequently sacrificed 45 minutes later.

Acetylcholine was extracted in eserinizd-acid-saline and quantitated using the guinea pig ileum (confer Materials and Methods, Section I of this thesis). MS1 itself at a bath concentration of 1 mg/ml, was tested and was not found to interfere with the ileal response to acetylcholine at a bath concentration of 0.001 gamma/ml.



### C. RESULTS

#### a. Regional Brain Acetylcholine Concentrations In Mus musculus CF1 Mice Treated With Methionine Sulfoximine (MSI).

Intraperitoneal administration of MSI at a dose of 100 mg/kg to CF1 mice caused a series of spontaneous, convulsive episodes (running fits) after a prolonged latency of approximately six hours. During this six hour interim, the mice exhibited progressive degrees of lethargy and behavioral depression. This was not sedation, however. The severe lethargy and depression toward the end of the six hour latency sporadically broke into convulsive episodes without an intervening stage of excitation.

The state of regional brain acetylcholine concentrations at the beginning and toward the end of this six hour preconvulsive period is indicated in Table 7 and Figure 12. The changes in acetylcholine at 45 minutes after 100 mg/kg MSI are not statistically significant but indicate, at least for the T, a possible decrease in acetylcholine concentration. Four and one half hours after this same dose of MSI (shortly before the onset of convulsions), T acetylcholine is significantly increased by 37% ( $P < .01$ ). Increases in acetylcholine of MD, 13%, and PM, 17%, were also found their levels of probability were not significant,  $P < 0.1$  and  $P < 0.2$ , respectively. There appears to be a trend here of a general increase in brain acetylcholine concentrations prior to the onset of convulsive episodes.

The effects of 25 mg/kg MSI, a non-convulsive dose, were also investigated at four and one half hours after i.p. injection. Behaviorally, no lethargy or depression was noted, in fact, this dose of MSI has been shown to increase exploratory behavior (Scudder, unpublished data). Neurochemically, T acetylcholine was increased by 32%. However, this increase was not

Experimental Treatment	Duration of Treatment	Regional Brain Ach, Gamma/Gram $\pm$ SE		
		T <sup>+</sup>	MD <sup>++</sup>	PM <sup>+++</sup>
Saline, i.p.	4.5 hours	$0.79 \pm .03$ (30/6)*	$1.16 \pm .07$ (30/6)	$0.94 \pm .01$ (20/4)
MSI 100 mg/kg, i.p.	4.5 hours	$1.08 \pm .07$ (24/5)	$1.31 \pm .07$ (24/5)	$1.10 \pm .01$ (20/4)
% change from saline control		+37**	+13	+17
Saline, i.p.	4.5 hours	$0.79 \pm .03$ (30/6)	$1.16 \pm .07$ (30/6)	$0.94 \pm .01$ (20/4)
MSI 25 mg/kg, i.p.	4.5 hours	$1.04 \pm .17$ (20/4)	$1.22 \pm .07$ (20/4)	$1.00 \pm .06$ (15/3)
% change from saline control		+32	+5	+6
Saline, i.p.	45 minutes	$0.92 \pm .04$ (10/2)	$1.15 \pm .27$ (10/2)	$0.98 \pm .19$ (10/2)
MSI 100 mg/kg, i.p.	45 minutes	$0.75 \pm .05$ (15/3)	$0.95 \pm .16$ (10/2)	$1.10 \pm .04$ (15/3)
% change from saline control		-18	-17	+12

Table 7. Effect of 100 mg/kg (i.p.) methionine sulfoximine (MSI) at 45 minutes and 4.5 hours after injection and of 25 mg/kg (i.p.) MSI at 4.5 hours post injection on ach concentrations (mean  $\pm$  SE, micrograms/gram) in three brain regions of adult, male Mus musculus CF1 mice.

+ Telencephalon  
 ++ Midbrain-Diencephalon  
 +++ Pons-Medulla

\* Total number of mice used/number of determinations

\*\* Statistically significant

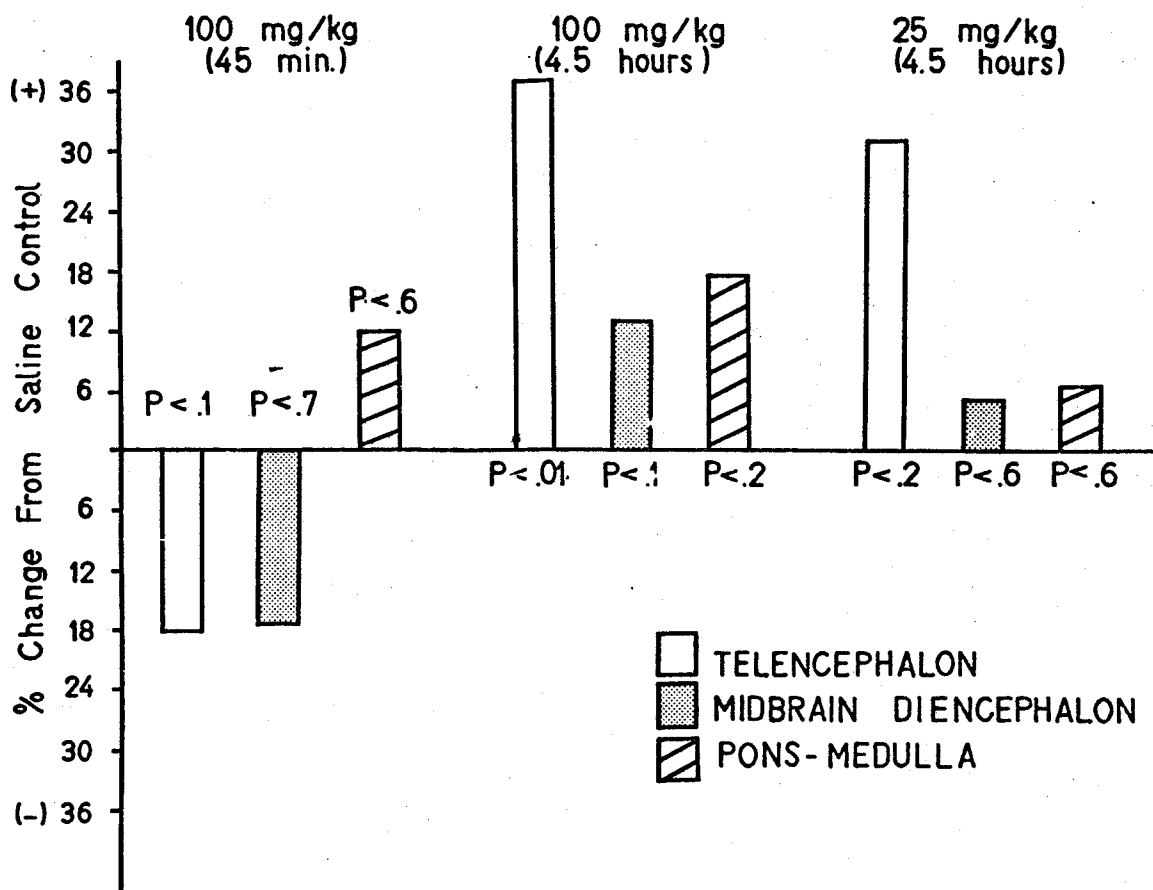


Figure 12. Percent change from saline, control, each concentration in three brain regions of adult, male *Mus musculus* CF1 mice 45 minutes and 4.5 hours after 100 mg/kg (i.p.) methionine sulfoximine (MSI) and 4.5 hours after 25 mg/kg (i.p.) MSI. P values are given for % changes of each brain part in all three experimental groups.

statistically significant ( $P < 0.2$ ). The minor increases in acetylcholine of M-D (5%) and P-M (6%) were not significant ( $P < 0.6$  for both). Again considering tendencies, the increase in brain acetylcholine four and one half hours after 25 mg/kg MSI seems restricted primarily to the T and is not very prominent in either MD or PM. Therefore, while both 25 mg/kg and 100 mg/kg doses of MSI seem to affect the T acetylcholine to the greatest extent, only the convulsive dose tends to appreciably alter the MD and PM acetylcholine concentrations; the non-convulsive 25 mg/kg dose affects these brain regions proportionately less.

b. Effect Of MSI On Hexobarbital Sleeping Times

Pilot studies were conducted to determine the effect of MSI on hexobarbital (75 mg/kg, i.p.) sleeping time. Compared to control values, sleeping times were increased by 32% at 45 minutes after 100 mg/kg MSI ( $P < .05$ ), 74% at four and one half hours after 100 mg/kg ( $P < .1$ ) and 34% at about four and one half hours after 25 mg/kg MSI ( $P < .2$ ). These studies indicate the ability of MSI to induce different degrees of susceptibility to hexobarbital depression. Pretreatment for 45 minutes with 100 mg/kg MSI caused a mild increased susceptibility to hexobarbital. At four and one half hours after this same dose of MSI, the animal exhibits a much greater susceptibility. Mice exposed to 25 mg/kg MSI four and one half hours responded to hexobarbital similarly to animals that had been exposed to 100 mg/kg MSI for 45 minutes.

c. Influence Of Age And Scopolamine HBr On MSI Toxicity

Age

During the course of the experiments it was noted that 100 mg/kg MSI caused a variable number of deaths even though spontaneous convulsions

were generally observed. The age of the mice may have influenced mortality. Upon testing the effect of age on MSI toxicity, it was found that 37% of a 90 day old group (average body weight of 33 grams) died, while only 17% of the 50 day old group (average body weight of 27 grams) were found dead. Therefore, older (or heavier) animals are more susceptible to the lethal effects of MSI than are younger mice.

#### Scopolamine HBr

In a separate pilot study, scopolamine, in a cumulative dose of 120 mg/kg (1/5 the calculated LD50), increased the lethal effect of 100 mg/kg MSI, in 62 day old mice by 100%. This treatment with scopolamine was, however, observed to delay or offset the convulsive effects of MSI.

#### D. DISCUSSION

Within 45 minutes of receiving a convulsive dose of MSI (100 mg/kg, i.p.) the acetylcholine levels of the telencephalon tended to decrease. The behavioral state of these mice was found to be that of lethargy and depression (Scudder, unpublished data). This behavioral appearance could be the result of too little effective cholinergic activity, for example, if MSI had a strong inhibitory effect on synthesis (Heath, 1966) or on the cholinergic receptor; on the other hand such apparent depression could result from hyperactivity of the cholinergic system, for instance, if MSI enhanced the conversion of bound to free acetylcholine (Tower and Elliott, 1953). In each of the above possibilities a decreased level of acetylcholine could be expected. The additional finding by the present author that 45 minutes after this dose of MSI there is a potentiation of hexobarbital sleeping time may be taken as an indication of a decreased state of biological activity (Bousquet, et al, 1965). However, in the absence of other available information no conclusions can be made as to whether cholinergic activity is increased or decreased at this time. But it does appear that, if an increased cholinergic activity is occurring, little biological activation is accompanying it. The mechanism for decreasing the acetylcholine may be associated with the mild depression of glutamine synthetase and glutamine transferase which has developed by this time after the injection of MSI (Lamar and Sellinger, 1965).

After a latency of 5 to 6 hours, this same dose of MSI (100 mg/kg, i.p.) results in the appearance of episodic running fits and associated convulsions. Prior investigators have established that acetylcholine levels at this time are either not changed (De Robertis, et al, 1967; and Gershoff and Elvehjem, 1951) or decreased (Peters and Tower, 1959; Tower, 1955; and

Tower and Elliott, 1953). Such changes in acetylcholine are expected during or immediately after any type of seizure (Richter and Crossland, 1949). Tower and Elliott (1953) demonstrated that, at the time of MSI induced convulsions, as well as anoxic or epileptic seizures, the ability of brain tissue to bind acetylcholine is markedly reduced. Naruse, et al (1960) subsequently related such conversion of bound to free acetylcholine with the initiation of seizure activity. But they further concluded that a high amount of acetylcholine should be present initially to predispose the animal to the convulsion. The results presented in this paper establish the fact that before initiating the convulsive seizure, possibly by causing a defect in binding (Tower and Elliott, 1953), MSI predisposes the animal to convulsion by increasing the brain acetylcholine concentrations. Four and a half hours after receiving 100 mg/kg, MSI, i.p., (which is shortly before the onset of convulsion), telencephalic acetylcholine is increased significantly; midbrain-diencephalon and pons-medulla show a marked tendency to increase acetylcholine concentrations, as well. Therefore, the mouse becomes more prone or more susceptible to convulse by virtue of its increased acetylcholine levels. As proof of their increased susceptibility to seizure, Scudder (unpublished data) found that mice treated in this manner would undergo seizure if exposed to even the mild, electric shock of a training apparatus. These findings further confirm the association made by Kurokawa, et al (1963), Naruse, et al (1960) and Takahashi, et al (1961) between high acetylcholine brain levels and a predisposition to seizure.

In view of this present work, the depression of acetylcholine binding by MSI seems to occur only at the onset of convulsions (and possibly during the preconvulsive period immediately after injection). Despite the increased susceptibility to seizure, the mice at this time appeared very

lethargic and behaviorally depressed. Scudder (unpublished data) confirmed this by finding that many behavioral parameters were markedly disrupted, including aggression and exploratory behavior. Furthermore, hexobarbital sleeping times were potentiated by 74%. This marked potentiation clearly distinguished the state of biological activity of these animals from that of stress mice (refer to Chapter VIII of this thesis). Consequently, it was concluded that these MSI treated animals were in a state of severe lethargy and depression in association with decreased central cholinergic activity.

The mechanism by which MSI induces this increase in acetylcholine is entirely unknown. The only other biochemical changes thus far reported during the preconvulsive period of MSI treated animals are that glutamine synthetase (converts glutamate plus ammonium to glutamine) and glutamine transferase (catalyzes glutamine breakdown to glutamate and ammonium) activities are practically abolished four hours post injection. The states of other enzyme systems have not yet been determined. Furthermore, the actual preconvulsive levels of glutamate, glutamine, GABA, ammonium, etc. have not been determined. From the available information, ammonium and glutamate should be building up due to the inhibition of glutamine synthetase. Gershenovich, et al (1963) have indicated, however, that excess ammonium can be removed by reacting with alpha-keto glutaric acid to form glutamate, but, since reacting with glutamate is the primary metabolic pathway for removing ammonia, and since MSI is also capable of inhibiting other enzymes (De Robertis, et al, 1967), indications point to the possibility that both ammonia and glutamate are building up during the preconvulsive period. The increased glutamate may result in the increased acetylcholine levels, since Peters and Tower (1959) concluded that glutamate somehow participates in



maintenance of acetylcholine-binding capacity. This maintenance may be maintained by the presence of ammonium which Quastel (1962) found to enhance conversion of bound to free acetylcholine possibly by its neuronal irritant property (Richter and Dawson, 1948). The binding capacity may, however, be effective enough to retain the acetylcholine until some disruptive triggering mechanism is introduced.

Such a triggering mechanism could take many forms. A strong sensory input could initiate the conversion of bound to free acetylcholine. In the presence of high brain ammonium the brain would be in a high state of irritability. This, together with the high acetylcholine levels, could generate a convulsive seizure. That sensory stimulation can initiate a seizure is evident from the studies of Wada and Ikeda (1966) on MSI-induced audiogenic seizures; also Scudder (unpublished data) demonstrated that even a normally mild electric shock can induce a seizure in an MSI-treated animal.

MSI itself could be responsible for automatically triggering a seizure, a fact well founded in the literature (Harris, 1964; Lamar and Sellinger, 1965; Sellinger and Rucker, 1966; and the present results). This spontaneous generation of seizures could occur in several ways. Anoxia has been found capable of triggering the release of acetylcholine (Tower and Elliott, 1953; Welsh, 1943). MSI may induce anoxia to trigger the seizure. This possibility is supported by the fact that anoxia necrosis of cortical tissue was found in MSI treated animals but only in association with seizure activity (Harris, 1964). Of course, such necrosis could have occurred as a result of the seizure activity and not as the precipitating event.

MSI may also trigger seizures by acting to disrupt the cellular lysosomes which have been shown to be associated with the initiation of

convulsions (Sellinger and Rucker, 1966). These authors found that disruption of lysosomes by MSI correlated temporally with the onset of convulsive episodes. This disruption was reversible, occurring only in animals that convulsed and only after the latency period characteristic of MSI-induced convulsions.

To summarize the above discussion, shortly after the i.p. administration of a convulsant dose of MSI, there appears a tendency for brain acetylcholine, especially of the telencephalic region, to decrease. This is associated with a mild to moderate behavioral and motor depression. The moderate prolongation of hexobarbital sleeping time in these animals indicates a low state of biological activity. This decrease in acetylcholine could be due to a number of effects of MSI: 1), inhibition of acetylcholine synthesis (Heath, 1966);, 2) inhibition of the cholinergic receptors; or 3) disruption of the acetylcholine binding mechanism (Tower and Elliott, 1953). Approximately four and a half hours after this same dose, the acetylcholine levels have increased significantly in the telencephalon, but less in the midbrain-diencephalon and pons-medulla. This increase is correlated with a marked lethargy and behavioral depression. Hexobarbital sleeping times are also markedly prolonged thereby indicating a high degree of biological inactivity. This increased acetylcholine points out the possibility that MSI does alter acetylcholine-binding primarily at the onset of convulsion but that MSI is also capable of priming an animal to convulsive seizures by increasing the amount of available acetylcholine. MSI could induce this increase in acetylcholine by causing glutamate to build up and in turn increase the acetylcholine-binding capacity; a similar increase in ammonium could increase the irritability of the brain. This would result in a seizure, when some triggering mechanism, possibly activated by MSI, initiates the conversion of

bound to free acetylcholine. The degree of applicability of this proposed mechanism for the increase in brain acetylcholine and for the induction of seizure in MSI-treated animals is consequent upon determinations of ammonium, etc, and of the activities of a host of related enzymes.

Apparently, as Lamar (1968) had concluded, MSI does not produce seizures by inhibiting glutamine synthetase alone, since this enzyme was blocked by subconvulsive doses and during the preconvulsive period (Lamar and Sellinger, 1965). The results presented in this paper indicate a possible necessity for the presence of a certain level of acetylcholine, in order for MSI to effect a convulsive. This has been proposed to be a factor in any type of seizure (Naruse, et al, 1960; Richter and Crossland, 1949; and Takahashi, et al, 1961). In these results a non-convulsive dose of MSI was shown to cause as marked an increase in telencephalic acetylcholine (though not statistically significant) as did the convulsive dose at the same time interval. Furthermore, the minor, non-significant increases in midbrain-diencephalon and pons-medulla, after the low dose of MSI, were very much reduced compared to the effect of the convulsant dose. In essence the only appreciable change in acetylcholine induced by the non-convulsant dose of MSI was an increase restricted to the telencephalon. Very possibly, in order for MSI to generate a seizure, a more general rise in brain acetylcholine is required. Such a general increase was apparently not reached by the lower dose.

Additional information suggesting the release of acetylcholine to be central to seizure activity in MSI treated animals was obtained in a pilot study with scopolamine. This anticholinergic drug delayed or offset the convulsive effect of MSI in agreement with similar findings by Wada and Ikeda

(1966). This retardation of seizure might be related to a decrease of brain acetylcholine by scopolamine (confer Chapter IX of this thesis). Interestingly enough, however, scopolamine enhanced the lethal effect of the convulsant dose of MSI. This unexpected finding is compounded by the fact that older mice are more susceptible to the lethal effects of MSI than are younger mice. Since scopolamine decreases acetylcholine availability (see Chapter IX) and since aging increases brain levels of acetylcholine (Crossland, 1951; and Crossland and Merrick, 1954), the similarity of the effects of age and of scopolamine on the lethality of MSI is at present inexplicable by a cholinergic hypothesis.

The behavioral appearance of mice, which received the nonconvulsive dose of MSI (25 mg/kg), is somewhat similar to that induced by an injection of an anticholinergic drug. Scudder (unpublished data) observed an increase in exploratory activity in MSI-treated animals, a behavioral state equally induced by scopolamine (Meyers and Domino, 1964; and Parkes, 1965). Similarly, the antiaggressive effect of scopolamine (Janssen, et al, 1960) is shared by MSI (Scudder, unpublished data). Furthermore, potentiation of hexobarbital sleeping, shown here to be a property of this low dose of MSI, is also characteristic of scopolamine and atropine (Giarmann and Pepeu, 1962; and Mensch and De Jongh, 1958). White and Rudolph (1968) consider such synergism with barbiturates a common characteristic of CNS depressants. Hence, MSI may be classed as a depressant resembling an anticholinergic drug in behavioral effects. The tendency of MSI to improve learning slightly does not contradict this suggested resemblance, since such an enhancement of learning has occasionally been reported for scopolamine as well (Bignami, 1964).

The disparity between the subconvulsive and preconvulsive effects of MSI and the effect of scopolamine on brain acetylcholine indicates, at

least, a different mechanism of action for each drug; MSI increases acetylcholine and scopolamine decreases it. The resultant effect of both is similar, however, i.e. disruption of cholinergic function. The similarity in effects of these 2 substances takes on additional significance, when it is realized that this low dose of MSI and scopolamine (after an hour) alters the cholinergic levels primarily in the telencephalic region of the brain. Aprison, et al (1968) related telencephalic cholinergic activity with states of behavioral excitation. This present finding emphasizes the importance of the cholinergic system, specifically of the telencephalic brain region, in exploratory behavior, the expression of aggression and states of biological activity.

It is concluded that, during the preconvulsive period and in subconvulsive doses, MSI has anticholinergic properties (as suggested by Heath, 1966), but that these properties are probably not due to an inhibition of synthesis and probably do not result from a mechanism similar to that of scopolamine. In appropriate circumstances, MSI can induce hypercholinergic activity in the generation of a seizure.

Correlative Discussion

Throughout this thesis it has become increasingly apparent that the cholinergic system plays a significant role in regulating the expression of a number of behaviors. The genetic studies revealed broad relationships between whole brain acetylcholine levels and behavior, and the findings of the more specific experimental studies clarified and modified these suggested cholinergic relationships.

Maximum exploration was exhibited by Mus mice which had low levels of whole brain acetylcholine. Higher acetylcholine levels (Onychomys and Peromyscus) or lower levels (Microtus) resulted in lower exploratory behavior. There appeared to be an optimum level of cholinergic activity for the maximum expression of exploration. Exposing Mus to a stressing experimental situation biochemically altered the brains of these mice. The telencephalon alone showed a significant increase in acetylcholine (indicative of hyperactivity of the cholinergic system). The stressed Mus, resembled Peromyscus and Onychomys mice, behaviorally, since their acetylcholine levels were increased. To further establish the relationship between cholinergic activity and exploration, scopolamine, an anticholinergic, returned this behavior to normal levels in the stressed Mus. Again, the telencephalon was implicated since, scopolamine was found to affect cholinergic function especially in this brain region. As mentioned above, the genetic data indicate that reduction of brain acetylcholine levels below that of Mus impairs exploration, e.g. in Microtus. This finding, obviously, must be qualified as to the brain region involved. A low dose of MSI in Mus (not

stressed as before) markedly reduces the cholinergic activity, specifically of the telencephalon, as does scopolamine; exploratory behavior is enhanced by both MSI (Scudder, unpublished data) and scopolamine (Meyers and Domino, 1964; Parkes, 1965). A larger dose of MSI depresses cholinergic activity throughout the brain. In this state exploratory behavior is virtually abolished, reminiscent of the low exploration and low total brain acetylcholine of Microtus. Therefore, perhaps there is some strength in the argument that exploratory behavior is inversely related to telencephalic cholinergic activity with the understanding that this cholinergic regulatory mechanism is dependant upon adequate activity of the cholinergic systems in the midbrain-diencephalon and pons-medulla.

The telencephalic cholinergic system also appears to be linearly related to the state of "emotionality" of an animal. Genetically, Peromyscus is representative of a higher "emotional" animal based primarily on its tendency to freeze during stress and to display stereotyped behavior (Karczmar and Scudder, 1967; Scudder, et al, 1966). This high brain acetylcholine animal is contrasted by the fearless and relatively non-freezing and non-stereotypic Mus and Microtus, both of which have low whole brain acetylcholine levels. When the relatively "unemotional" Mus are stressed, the telencephalic acetylcholine is increased and there is not only a depression of exploration but also an appearance of freezing behavior, normally not characteristic of this genus. Scopolamine reestablishes the behavioral profile of the stressed Mus by abolishing the freezing behavior. Specifically decreasing telencephalic cholinergic function in a normal Mus with a low dose of MSI does not facilitate the freezing behavior but depresses it even further. Decreasing the telencephalic cholinergic system is as effective in depressing freezing

behavior (or "emotionality") as is general-depression of the entire central cholinergic system. Therefore, states of periodic immobility, as a component of "emotionality", are directly related to the degree of cholinergic activity primarily in the telencephalon.

Stress seems to alter the behavioral profile of Mus partly as a function of telencephalic cholinergic activity and partly as a function of serotonergic and adrenergic activities. Possibly, due to these biochemical alterations, stress induces Mus to adopt behaviors resembling those of Peromyscus and Onychomys. Speculatively then, if the type of behavioral expression does depend upon the level of activity of the cholinergic system, Peromyscus and Onychomys mice should adopt the behavioral profile of Mus after receiving scopolamine or a low dose of MSI. An indication that this may be the case was observed by Scudder (unpublished data). He found the scopolamine did increase the exploratory behavior of Onychomys and Peromyscus mice.

The relationship of the cholinergic system to aggressive behavior is more complex according to the data presented in this thesis. This complexity stems from the fact that 2 cholinergic components seem to be involved with isolation induced aggression; one component resides in the midbrain-diencephalon, the other in the telencephalon. Isolation of a non-aggressive mouse for an appropriate period of time results in a highly aggressive animal. The only central cholinergic alteration occurring is a decrease in the midbrain-diencephalon acetylcholine concentration. However, the antiaggressive effect of scopolamine was found to correlate with a reduction in telencephalic cholinergic activity. The reduction of aggression by the low dose of MSI further supports the importance of the telencephalon



in aggression, since this dose of MSI also selectively depresses cholinergic activity in this brain region. Based on a theory proposed by Scott and Fredericson (1951), the cholinergic depression in the midbrain-diencephalon reflects a disruption of the process of habituation to incoming stimuli. The aggression-generating stimuli can now stimulate the telencephalon to activate a proposed innate aggressive behavior. The disruption of this aggressive behavior by scopolamine does apparently occur in the telencephalon but its point of interference is conjectural. It could prevent cortical reception of the triggering stimuli or it could hinder the organization of sensory information which integrates the actual act of the animal (Longo, 1966).

The genetic studies reported here shed little light on the role of the cholinergic system in aggression, since whole brain levels of acetylcholine were determined which would mask subtle, regional differences in acetylcholine, which are apparently of vital importance in aggression. However, in terms of total brain acetylcholine concentration an optimum level of acetylcholine exists for maximum aggressive behavior. Mus mice display the optimum acetylcholine levels and the maximum aggressiveness. Higher brain acetylcholine, e.g. Peromyscus or Onychomys, as well as lower acetylcholine, as in Microtus, are equally disruptive of aggressiveness. Possibly, these whole brain levels of acetylcholine might be more indicative of telencephalic acetylcholine since this region makes up the bulk of the brain. With this assumption, the optimum cholinergic levels would apply to the telencephalic component of aggressive behavior. Theoretically, at very low doses either a cholinomimetic or a cholinolytic could enhance aggression depending upon whether the animal is above or below the optimum

telencephalic level of cholinergic functioning. However, larger doses of either type of drug would tend to disrupt aggressiveness. Scudder (unpublished data) presented preliminary evidence that scopolamine, at very low doses, will enhance aggression but antagonize it at higher doses.

Learning ability, on the basis of the genetic study, was also found to be related to an optimum cholinergic activity. The whole brain acetylcholine levels of Mus mice was apparently best suited for maximum learning performance. The higher acetylcholine levels in Peromyscus, Onychomys and Perognathus were associated with poorer learning ability, as were the lower acetylcholine levels of Microtus. Experimentally, animals not allowed to solve a training problem were found to increase their telencephalic cholinergic activity. This could be interpreted to reflect a continually active cholinergic system, primarily in the telencephalon, during the course of learning the correct response. It would be of paramount importance to test the degree of learning ability of such an animal periodically during exposure to this insolvable training procedure. The genetic study indicates that increasing cholinergic activity above an optimum level disrupts learning performance. This was also suggested by the studies of Bovet, et al, (1969). Therefore, theoretically, learning performance should tend to improve during the initial exposure to the stressful environment but, later, at a certain level of telencephalic cholinergic activation, learning ability decreases. This point of depressed learning may accompany a high degree of emotionality in the stressed animal. This latter suggestion is based on the finding that the highly emotional Peromyscus mice are poorer learners than the relatively unemotional Mus. The depression of learning also corresponds to the decrease in the exploratory behavior of

the stressed mice. This is also suggested by the close parallelism of these 2 behaviors intergenerically, i.e. both are very low in Microtus, with low acetylcholine concentration, but reach a maximum in the Mus mice, which have higher acetylcholine levels than Microtus; then learning and exploration decline again in Peromyscus and Onychomys, both of which have very high brain acetylcholine. Further support for this relatedness is obtained from the fact that low doses of MSI enhance both exploration and learning ability. Although a casual relationship between these 2 behaviors is placed in question by scopolamine's ability to enhance exploration but to depress learning, the telencephalic cholinergic system does appear intimately involved in the process of learning.

On the basis of the effects of the convulsant and the non-convulsant dose of MSI, it appears that an increase in telencephalic acetylcholine, but not in the rest of the brain, is not sufficient to allow for MSI-generated convulsions. The larger convulsant dose also increases acetylcholine in the midbrain-diencephalon as well as the pons-medulla, but this additional effect is not shared by the smaller, non-convulsant dose. Intergenerically, seizure susceptibility is inversely related to whole brain acetylcholine levels, i.e. the lower the acetylcholine, the more prone the mouse is to electoshock seizure. This seems contrary to the findings with MSI which is in agreement with the conclusions of other investigators (Naruse, et al, 1960). Generally, high brain acetylcholine is associated with increased predisposition to convulsion. One possible explanation for this seemingly, anomalous intergeneric data is that the acetylcholine levels are paralleled by norepinephrine and serotonin, both of which have been implicated in prolonging electroshock latency (Scudder, et al, 1966). Also, the intergeneric

levels of acetylcholine may reflect the presence of cholinergic inhibitory or modulatory neuronal circuits which may also partake in restricting the electroshock current from generating a seizure (Wooley, et al, 1963). A more definite explanation for this association of high acetylcholine with low seizure susceptibility may be revealed by studies of brain parts of these genetically unique mice.

The behavioral responses considered in this paper - exploration, habituation, avoidance, fighting, periodic immobility (freezing), stereotypy, and apparent behavioral withdrawal - are all patterns of the organism's behavior which relate to the environmental challenges (Scott and Fredericson, 1951). Each of these was found to involve a cholinergic component, as part of a system of interacting neurochemical sub-systems. The cholinergic element appears to be central to the expression of each of these behavioral responses.

While it was possible to relate certain behaviors (exploration, "freezing", avoidance learning and emotionality) to cholinergic activity specifically within the telencephalon, only more refined techniques and further subdivision of the telencephalic components will enable investigators to conclude whether the expression of these various behaviors involves activation of the same or different cholinergic circuits. Other behaviors, such as aggression, were found to involve both a telencephalic as well as a midbrain-diencephalic, cholinergic component. This behavior was found to occur in association with a certain level of telencephalic cholinergic activity in accord with the optimum level theory (Crossland, 1960; Irwin, 1968; Malmö, 1966; Reeves, 1966; and Roberts, 1966). Therefore, the present findings are consonant with the theory that expression of certain adaptive

responses depends upon the patency of the cholinergic neurons (Bennett, et al, 1958). However, since little is known about the neural dynamics of the central nervous system, no decision can, at present, be made as to how - by inhibition of other neurons or by an excitatory action - the cholinergic system effects its control on adaptive behavior. Carlton (1963) proposed it to have a purely regulatory function, modulating or monitoring the neuronal subunits which would then generate a particular behavior. Such a regulatory function is supported by the involvement of the cholinergic system with the reticular formation (Collier and Mitchell, 1966 a and b; Rinaldi and Himwich, 1955) which has been shown to exert a modulatory control over behavioral response to stimuli (Rosvola and Mishkin, 1961) as well as over sensory input (Carlton, 1968). In this capacity it could serve to influence or bias "decisions" of the integrating brain centers. In addition to regulating behavioral response, the cholinergic system could also partially comprise the neuronal subunits themselves, responsible for organizing and carrying out the behaviors (Aprison, 1968; Deutsch and Lutzky, 1967). Regardless of its exact mechanism, the apparent participation of the cholinergic system in the expression of vital behaviors, as shown in this thesis, points out the necessity of accounting for its level of activity in any future behavioral studies.

## CHAPTER XII

SUMMARY

Acetylcholine concentrations of whole mouse brain and brain parts were determined using a acid-eserine-saline method of extraction and a guinea pig ileum bioassay.

The present work is unique in its determination of whole brain levels of acetylcholine across seven genera (fourteen strains) of mice. Intergenerically, brain acetylcholine was directly correlated with whole brain serotonin and norepinephrine concentrations. Whole brain levels of acetylcholine were also correlated with electroconvulsive shock latency and several behavioral traits such as exploration, aggression, stereotypy, "freezing", learning and locomotor activity. The body weights and brain weights of the fourteen varieties of mice used in this study were also presented.

The investigations reported in this thesis on environmentally-induced alterations of acetylcholine concentrations and drug effects on acetylcholine concentrations in discrete brain parts of the laboratory mouse are also unique to this thesis. The brain parts involved were telencephalon, midbrain-diencephalon, and pons-medulla.

Exposure of the laboratory mouse to a shock-avoidance learning procedure did not alter the acetylcholine levels in any of the three brain parts; but, an animal not allowed to escape from the shocking apparatus exhibited characteristic behaviors and a selective increase of the telencephalic acetylcholine. This increased acetylcholine in the stressed mice was discussed in terms of a theory of increasing cholinergic activation of the brain during changes of states of behavior.

The investigation of the effects of isolation on the central cholinergic system reported here is also an unprecedented study. Isolation was found to be accompanied by a decrease in acetylcholine levels specifically of the midbrain-diencephalon. This was interpreted to represent a decrease in cholinergic activity and was discussed in relation to the process of habituation. Also characteristic of isolated mice is a reduction in weight of the telencephalic and of the pons-medullary brain regions but not of the midbrain-diencephalic area.

Another previously unreported effect is the modification of regional brain acetylcholine levels of isolated animals by an antiaggressive dose of scopolamine. Using isolated laboratory mice, specific reductions in acetylcholine concentrations were found to occur in both the telencephalon and the midbrain-diencephalon at 15 minutes after 2 mg/kg (i.p.) scopolamine HBr. However, 60 minutes after this same dose, at which time the anti-aggressive effect of scopolamine is maximal, only the telencephalic acetylcholine was reduced. The relationship of the central cholinergic system to scopolamine and its ability to depress aggressive behavior is discussed.

The present study is further unique in its investigation of the preconvulsive effects of methionine-sulfoximine (MSI) on the central cholinergic system. Forty-five minutes after a convulsive dose of MSI (100 mg/kg, i.p.), brain acetylcholine levels are below control values. However, shortly before the onset of seizure (four and one half hours after injection) an increase in telencephalic and pons-medullary acetylcholine is found. Four and one half hours after a non-convulsant dose of MSI (25 mg/kg, i.p.) only the telencephalic acetylcholine was increased. The necessity for increased brain levels of acetylcholine for the occurrence of seizure activity

is discussed with reference to MSI-induced seizures as well as to general seizure activity. A possible anticholinergic effect of MSI was also discussed in terms of its behavioral effects and its effects on regional brain levels of acetylcholine.



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APPROVAL SHEET

The dissertation submitted by Thomas J. Sobotka has been read and approved by five members of the faculty of Loyola-Stritch School of Medicine.

The final copies of the dissertation have been examined by the director of the examining committee and the signature which appears below verifies the fact that all necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 19, 1969  
Date

Charles L. Schneider  
Signature of Advisor